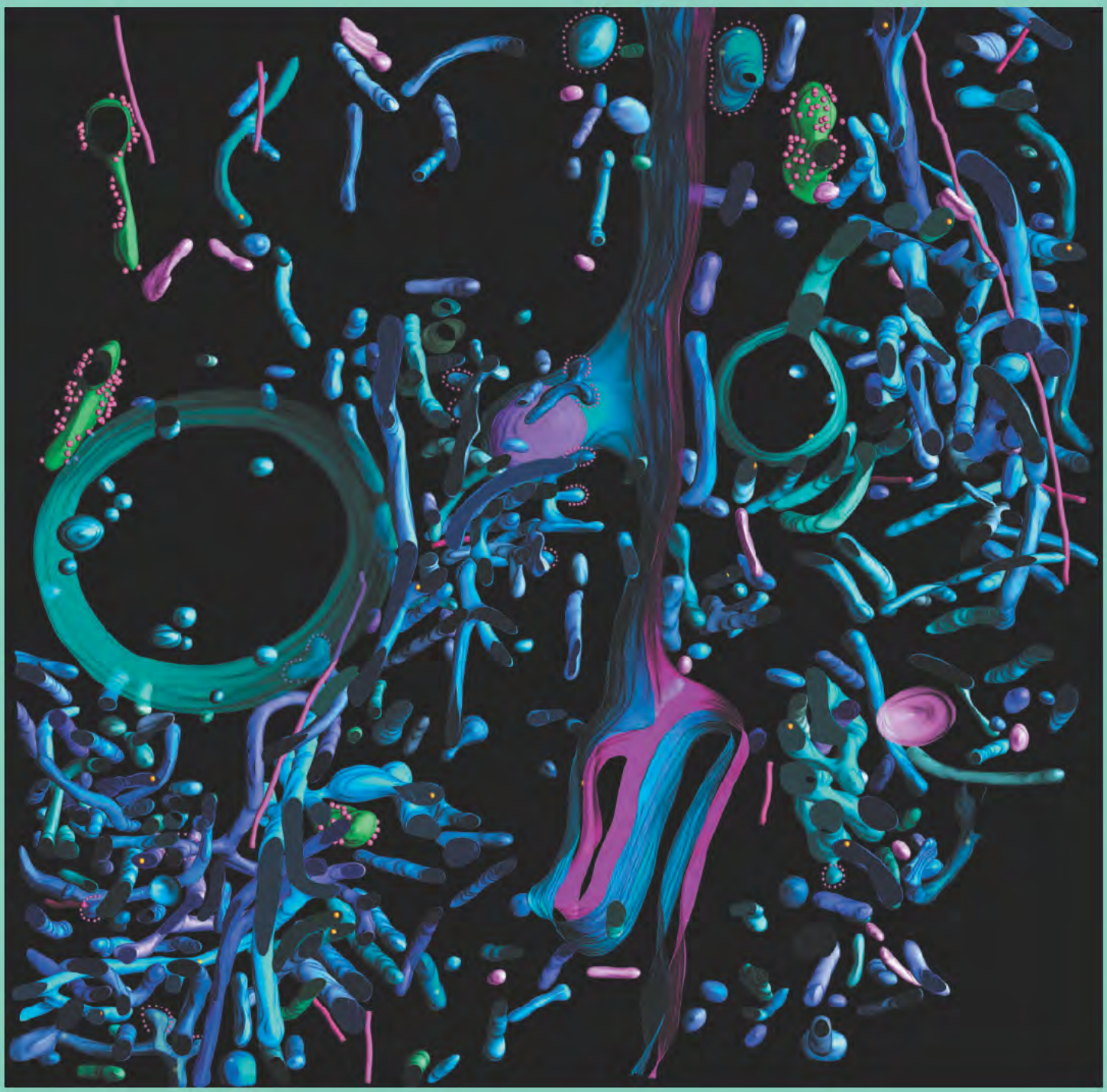

CALTECH Biology Annual Report 2007

Legend for the Front Cover Illustration

3D model derived by electron tomography of gold-labeled IgG fragments transported in proximal small intestinal cells by the neonatal Fc receptor. Transport vesicles (blue shades); microtubules (pink straws); lysosomes (violet); rough ER (green with scarlet ribosomes); mitochondria (bright green); lateral intercellular space membranes (purple and blue); clathrin coats (small red spheres around blue vesicles/buds); gold-labeled ligands (gold spheres). See abstract 379 by Wanzhong He, Grant J. Jensen, J. Richard McIntosh and Pamela J. Bjorkman.

Division of Biology

California Institute of Technology

Pasadena, California

Annual Report
2006 - 2007

BIOLOGY

Yolanda Duron, Annual Report Coordinator

I would like to express my gratitude to Stephanie Canada for her invaluable help in the preparation of this report.

Research Reports

Biological research summarized in this report covers the time period from June, 2005 through July, 2006. The annual report is not intended to serve as an official forum, since some portions of the research listed in this report have not yet been published. When referring to an individual abstract(s), special permission must be obtained from the investigator.

References to published papers cited throughout the report are listed at the end of each individual research report.

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FACILITIES

INTRODUCTION

100 Years Ago: 1907
From the Throop Institute Bulletin number 36, May, 1907
"In beauty and healthfulness, in the culture of its homes, and in its high social and moral tone, Pasadena has no superior on the Pacific Coast…"

In 1907 Throop offered a total of seven college-level classes in Biology: Vertebrate Anatomy and Physiology; Vertebrate Embryology; Systemic Study of Vertebrates; General Ornithology; Economic Entomology; Plant and Animal Ecology; and Bacteriology. These represented the interests of the two members of the Biology faculty, the well-known mammalogist and ornithologist Joseph Grinnell (1877-1939), who was Professor of Biology; and hygiene expert Ernest Bryant Hoag (1868-1924), Lecturer in Biology.

75 Years Ago: 1932
From the Bulletin of the California Institute of Technology volume LXI, number 137, 1932:
"A Department of Biology, rather than the traditional departments of Botany and Zoölogy, has been established, in order to emphasize the unity of the phenomena of living organisms… no attempt is made to cover at once the whole science of biology, but rather efforts are concentrated on the development of those of its branches which seem to offer the greatest promise as fields of research…"

Among the postdoctoral fellows in Biology Division in 1932 were Cyril Darlington, Curt Stern, Berwind Kauffmann, Barbara McClintock and George Beadle.

50 Years Ago: 1957
From Biology 1957, the 1957 Annual Report:
"A major addition to the faculty of the Division was arranged for in the appointment of Robert L. Sinsheimer of Iowa State College as Professor of Biophysics."

From Biology News Notes No. 8, May 1, 1957:
"A.H. Sturtevant has been awarded the Kimber Genetics Award of the National Academy of Sciences for achievement in the science of genetics… The presentation was made at the annual meetings of the Academy… During the meetings a symposium on genetics and radiation hazards was held, with G.W. Beadle as chairman. Max Delbrück spoke on the nature of genetic material.

25 Years Ago: 1982
From the CALTECH Biology Annual Report 1982:
"As our cover proclaims, this year was an exceptional one, with the award of the 1981 Nobel Prize in Physiology and Medicine to Professor Roger Sperry… Roger received half of the 1981 prize in recognition of his contributions to understanding the function of the human brain - specifically his work on the functional specialization and separateness of the two hemispheres… His friends and colleagues in the Division of Biology celebrated with a party on April 6, 1982…"

Assistant Professor of Biology Sarkis Mazmanian, joined the Biology Division faculty in the fall of 2006. Sarkis' research focuses upon the molecular mechanisms of the interactions between microbes and animals, emphasizing areas related to human health and disease. His laboratory studies bacteria, not as pathogens, but as organisms in a symbiotic relationship that is critical for the health of their animal hosts. He and his laboratory are pursuing such questions as: What are the molecular components of the host immune response that recognize and respond to the immunomodulatory signals of symbiotic bacteria? What are the cellular and molecular mechanisms that mediate protection to immune pathologies such as colitis and asthma? How can these novel molecular pathways provide insight into the proper functioning of the immune system and allow for the development of therapies against immune-based diseases? To address these questions, Sarkis uses biochemical and genomic methods.

FERGUSON AWARD, 2006

Jensen **Murphy** **Koch**

Dr. Gavin Murphy is the winner of the Ferguson Award for the 2006-2007 Academic Year. This award goes to the student who is judged by the faculty to have produced the best Ph.D. thesis over the past year. Dr. Murphy performed his graduate studies in the laboratory of Professor Grant Jensen. His thesis described electron cryotomographic imaging studies of intact bacterial cells and purified protein complexes. His highest-profile paper reported the first three-dimensional structure of a complete bacterial flagellar motor to about 7 nm resolution. The results were notable both as landmark demonstrations of advancing cryoelectron microscopy technology and as specific structural discoveries.

PROFESSORIAL AWARDS, 2006 - 2007

Bren Professor of Psychology and Neuroscience, Ralph Adolphs, was awarded the ASCIT award for excellence in undergraduate mentorship, and was also awarded the Simons Foundation Research Grant for studies on autism.

James G. Boswell Professor of Neuroscience, Richard A. Andersen, gave the Langer Lecture at the University of Toronto in 2007; also, was elected to the Institute of Medicine of the National Academies.

Roger W. Sperry Professor of Biology, David J. Anderson, was elected to the National Academy of Science in 2007.

Max Delbrück Professor of Biology, Pamela J. Bjorkman, in 2006 gave the Gairdner Foundation Lecture in Edmonton and Calgary Canada, Gairdner Laureate; and in 2007, gave the Keynote Address, SBNet Structural Biology meeting, Tällberg, Sweden.

Albert Billings Ruddock Professor of Biology, Marianne Bronner-Fraser, was elected/awarded as follows: President-Elect, Society for Developmental Biology; Board of Directors, Gordon Research Conferences; NIDCR Council; Editor: Developmental Biology, Journal of Cell Biology, Molecular Biology of the Cell; Board of Directors, Society for Developmental Biology; Scientific Advisory Board - March of Dimes; Scientific Advisory Board - Sontag Foundation; and the Javits Award, NINDS (2002-2009).

Associate Professor of Biology, **David Chan**, was awarded the Mitochondrial Research Society Young Investigator in 2007 and became the Ellison Medical Foundation Senior Scholar.

Assistant Professor of Biology and Applied Physics-Bren Scholar, Michael Elowitz, was awarded the Packard Fellowship in November, 2006; and was also awarded the MacArthur Fellowship in 2007.

Lois and Victor Troendle Professor of Cognitive and Behavioral Biology and Professor of Computation and Neural Systems; Executive Officer for Neurobiology, Christof Koch, became a Fellow of the American Academy of Arts and Sciences.

Lawrence A. Hanson Jr. Professor of Biology and Computation and Neural Systems, Gilles J. Laurent, gave the Ernst Florey Lecture at the University of Gottingen in 2007.

Bren Professor of Biology, Henry A. Lester, received the K.S. Cole Award of the Biophysical Society at its Annual Meeting in March 2007. He was also elected President of the Biophysical Society for the 2008-2009 year.

Assistant Professor of Biology, Sarkis K. Mazmanian, was recipient of the Career Development Award and the Crohn's and Colitis Foundation Searle Scholars Program Award.

George W. Beadle Professor of Biology and Division Chair, Elliot Meyerowitz, shared the Balzan Prize in 2006 with C.R. Somerville of the Carnegie Institution. It was given in the area of plant molecular genetics, "for their joint efforts in establishing *Arabidopsis* as a model organism for plant molecular genetics." He was also the Keynote Lecturer at the Gordon Conference on Quantitative Genetics and Genomics in February 2007.

Albert Billings Ruddock Professor of Biology, Ellen V. Rothenberg, was awarded the ASCIT Teaching Award, and was appointed to the Albert Billings Ruddock Professorship.

Howard and Gwen Laurie Smits Professor of Cell Biology, Alexander Varshavsky, has received the 2007 Schleiden Medal from Deutsche Akademie der Naturforscher Leopoldina, Germany, and the 2007 Weinstein Distinguished Lectureship from the American Association for Cancer Research.

PASSING

Felix Strumwasser (1934-2007)

Dr. Felix Strumwasser died on April 19, 2007 after a long career studying neuronal function, behavior, and circadian rhythms. He was on the faculty of the Division of Biology at the California Institute of Technology from 1964 to 1984. Born in Port of Spain, Trinidad, British West Indies, he received his education at UCLA, earning a B.A. in Zoology at the young age of 19, in 1953, and a Ph.D. in Neurophysiology and Zoology with T.H. Bullock, in 1957. During a postdoctoral period at the National Institute of Mental Health and the Walter Reed Army Institute of Research, he obtained long-term recordings from the brain of ground squirrels. These achievements enabled him to study circadian rhythms and even hibernation at the single-neuron level in mammals. He recognized the need for simpler and more accessible nervous systems and helped to pioneer the use of the marine mollusk *Aplysia californica* as an important preparation for neurobiology.

These studies caught the attention of the legendary Caltech invertebrate neurophysiologists, Antonie van Harreveld and C.A.G. Wiersma, who persuaded Felix to join the Caltech faculty as Associate Professor of Biology. He set up a large seawater aquarium system on the 3rd floor of Kerckhoff, requiring regular deliveries by tanker truck from the Pacific Ocean. Felix stayed at Caltech for twenty years where he was a pioneer in the application of cellular and biochemical techniques to single identified neurons. He and his colleagues studied single neuron activity, neuronal and hormonal bases of behavior, especially the nature and activity of the egg-laying hormone, and the neural mechanisms of sleep and circadian rhythm.

In 1984 he left Caltech for the Boston University School of Medicine, moving in 1987 to the Marine Biological Laboratory in Woods Hole, and then in 1992, to the Uniformed Services University of Health Sciences. From 1993 to 1994 he also served as Program Director for Neuronal and Glial Mechanisms at the National Science Foundation.

The Biology Division hosted the following lectures:

Weigle Lectures

Roger Tsien
March 14, 2007
HHMI, Department of Pharmacology
UC San Diego
"Building molecules to image synaptic plasticity and tumors *in vivo*"

George Klein
April 16, 2007
Microbiology and Tumorbiology Center
Karolinska Institute
"Our multiple defenses against cancer"

Wiersma Lectures

Martin Heisenberg
May 31, 2007
"Genetic Intervention in the *Drosophila* Brain Links Circuitry to Behaviour"

Richard D. Mooney
June 6, 2007
"Phantom Feedback, Mirror Neurons and Brain Pathways for Vocal Learning"

Photos from the Biology Division Retreat at Lake Arrowhead Resort,
November 3-5, 2006, taken by Elliot Meyerowitz

Photos from the Delbruck Centennial, A Celebration of the 100th Anniversary of the birth of Max Delbruck, held January 8, 2007. Photos taken by Bob Paz

Photos from Eric Davidson At 70: A Symposium on the Regulatory Genome, April 13, 2007.
Photos courtesy of Anne E. Cutting, Tom Soulanile, and Morris Maduro.

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INSTRUCTION AND RESEARCH

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Raymond J. Deshaies, *Executive Officer for Biology*
Christof Koch, *Executive Officer for Neurobiology*

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George Beadle Professor of Biology

Seymour Benzer, Ph.D., D.Sc h.c.
Crafoord Laureate
James G. Boswell Professor of Neuroscience (Active)

Charles J. Brokaw, Ph.D.,
Professor of Biology

John J. Hopfield, Ph.D.
Roscoe G. Dickinson Professor of Chemistry and Biology

Ray D. Owen, Ph.D., Sc.D h.c.
Professor of Biology

Jean-Paul Revel, Ph.D.
Albert Billings Ruddock Professor of Biology

Melvin I. Simon, Ph.D.
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James G. Boswell Professor of Neuroscience

David J. Anderson, Ph.D.[1]
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Giuseppe Attardi, M.D.
Grace C. Steele Professor of Molecular Biology

David Baltimore, Ph.D., D.Sc h.c., D.Phil.h.c., *Nobel Laureate*, *Robert Andrews Millikan Professor of Biology*

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[1] *Joint appointment with Howard Hughes Medical Institute*

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[1]*Joint appointment with Howard Hughes Medical Institute*

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Summary: This laboratory's research centers on the early formation of the nervous system in vertebrate embryos. The peripheral nervous system forms from two cell types that are unique to vertebrates: neural crest cells and ectodermal placodes. We study the cellular and molecular events underlying the formation, cell lineage decisions and migration of these two cells types. The neural crest is comprised of multipotent stem-cell-like precursor cells that migrate extensively and give rise to an amazingly diverse set of derivatives. In addition to their specific neuronal and glial derivatives, neural crest cells can also form melanocytes, craniofacial bone and cartilage and smooth muscle. Placodes are discrete regions of thickened epithelium that give rise to portions of the cranial sensory ganglia, as well as form the paired sense organs (lens, nose, ears). Placodes and neural crest cells share several properties including the ability to migrate and to undergo an epithelial to mesenchymal transition. Their progeny are also similar: sensory neurons, glia, neuroendocrine cells, and cells that can secrete special extracellular matrices.

We focus on studying the mechanisms underlying the induction, early development and evolution of the neural crest and placodes. This research addresses fundamental questions concerning cell commitment, migration and differentiation using a combination of techniques ranging from experimental embryology to genomic approaches to novel gene discovery and identification of gene regulatory regions. These studies shed important light on the mechanisms of neural crest and placode formation, migration and differentiation. In addition, the neural crest and placodes are unique to vertebrates. In studying the evolution of these traits, we hope to better understand the origin of vertebrates.

Because these cell types are involved in a variety of birth defects and cancers such as neurofibromatosis, melanoma, neuroblastoma, our results on the normal mechanisms of neural crest development provide important clues regarding the mistakes that may lead to abnormal development or loss of the differentiated state.

1. Lens-derived Semaphorin3A regulates sensory innervation of the cornea

Peter Y. Lwigale, Marianne Bronner-Fraser

The cornea, one of the most highly innervated tissues of the body, is innervated by trigeminal sensory afferents. During development, axons are initially repelled at the corneal margin, resulting in the formation of a circumferential nerve ring. The nature and source of guidance molecules that regulate this process remain a mystery. We find that the lens, which immediately underlies the cornea, repels trigeminal axons *in vivo* and *in vitro*. Lens ablation results in premature, disorganized corneal innervation and disruption of the nerve ring and ventral plexus. We show that this effect is mediated by Semaphorin3A (Sema3A) synthesized by the lens that interacts with Neuropilin-1 (Npn1) receptors on trigeminal axons. Inhibition of Sema3A-Npn1 signaling abrogates axon repulsion by the lens and cornea *in vitro* and phenocopies lens removal *in vivo*. These results demonstrate that lens-derived Sema3A mediates initial repulsion of trigeminal sensory axons from the cornea and is necessary for the proper formation of the nerve ring and ventral plexus.

2. Axial patterning in cephalochordates and the evolutionary origin of the gastrula organizer

Jr-Kai Yu, Marianne Bronner-Fraser, Nick Holland[*], Linda Holland[*]

The organizer of the vertebrate gastrula, which induces and patterns dorsal axial structures, expresses a characteristic suite of transcription factors and secretes antagonists of BMP-, Nodal- and Wnt-signaling pathways. Despite long-standing interest, the evolutionary origin of the organizer remains unclear. We show that the gastrula of the cephalochordate amphioxus expresses orthologues of organizer genes in patterns reminiscent of their deployment in vertebrates. Genes involved in dorso/ventral (D/V) patterning include key transcription factors that are expressed similarly to their vertebrate orthologues, as well as signals that are expressed dorsally (*Nodal, Lefty, Chordin,* and ADMP) or ventrally (e.g., the BMP signaling modulators BAMBI and Tolloid). Genes involved in anterior/posterior (A/P) patterning include a suite of *Wnt* genes expressed posteriorly around the blastopore and several Wnt antagonists, most expressed anteriorly, consistent with a role for *Wnts* in A/P patterning. These findings suggest that both cephalochordates and vertebrates use homologous gene networks for both D/V and A/P patterning. In light of recent phylogenetic analyses placing cephalochordates basal in the chordate lineage, it is likely that separate D/V and A/P signaling centers, as proposed for chick and mouse, may already have been present in ancestral chordates.

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3. Snail2 directly represses *cadherin6B* during epithelial-to-mesenchymal transitions of the neural crest

Lisa Taneyhill, Ed Coles, Marianne Bronner-Fraser

The neural crest, a transient migratory population of cells, forms craniofacial skeleton, peripheral nervous system, and other derivatives. The transcriptional repressor Snail2 is thought to be critical for the epithelial-to-mesenchymal transition that promotes neural crest delamination from the neural tube; however, little is known about its downstream targets. To this end, we depleted avian Snail2 protein in the premigratory neural crest using morpholino antisense oligonucleotides and examined effects on potential targets by quantitative PCR. Several dorsal neural tube genes were upregulated by alleviation of Snail2 repression; moreover, the cell adhesion molecule *cadherin6B* was de-repressed as early as 30 minutes. *Cadherin6B* has a reciprocal expression pattern to Snail2, and its regulatory region contains three pairs of clustered E boxes, representing putative Snail2 binding sites. Furthermore, *in vivo* and *in vitro* biochemical analyses demonstrate that Snail2 directly binds to these sites. These results are the first to describe a direct target of Snail2 repression *in vivo* during the epithelial-to-mesenchymal transition that characterizes neural crest development.

4. Neuropilin 2/Semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation

Laura Gammill, Constanza Gonzalez, Marianne Bronner-Fraser

In the head of vertebrate embryos, neural crest cells migrate from the neural tube into the presumptive facial region and condense to form cranial ganglia and skeletal elements in the branchial arches. We show that newly formed neural folds and migrating neural crest cells express the *neuropilin 2* (*npn2*) receptor in a manner that is highly conserved in amniotes. The repulsive npn2 ligand *semaphorin (sema) 3F* is expressed in a complementary pattern in the mouse. Furthermore, mice carrying null mutations for either *npn2* or *sema3F* have abnormal cranial neural crest migration. Most notably, "bridges" of migrating cells were observed crossing between neural crest streams entering branchial arches 1 and 2. In addition, trigeminal ganglia fail to form correctly in the mutants and are improperly condensed and loosely organized. These data show that npn2/sema3F signaling is required for proper cranial neural crest development in the head.

5. Evolutionary conservation of cell migration genes: From nematode neurons to vertebrate neural crest

Yun Kee, Byung Joon Hwang[1], Paul Sternberg[2], Marianne Bronner-Fraser

Cell migration is essential during embryogenesis and recurs in cancer metastasis. Because migratory cells share common properties, we hypothesized that genetic networks involved in cell migration may be conserved between nematodes and vertebrates. To test this, we performed a comparative genomic analysis to identify vertebrate orthologs of the genes required for hermaphrodite-specific neuron (HSN) migration in *C. elegans* and examined their expression and function in vertebrate neural crest cells. The results demonstrate a high degree of conservation of regulatory components in long-range migrations between evolutionarily far-distant animals. Although the neural crest is a vertebrate innovation, our study suggests that neural crest migration might have evolved by utilizing a cell migration program already present in the common ancestors of vertebrates and invertebrates.

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6. *Spalt4* mediates invagination and otic placode gene expression of cranial ectoderm

Meyer Barembaum, Marianne Bronner-Fraser

Vertebrate placodes are regions of thickened head ectoderm that contribute to paired sensory organs and cranial ganglia. We demonstrate that the transcription factor *spalt4* is broadly expressed in chick preplacodal epiblast and later resolves to otic and olfactory placodes. Ectopic expression is sufficient to induce invagination of non-placodal ectoderm and prevents neurogenic placodes from contributing to cranial ganglia. Conversely, loss of *spalt4* function in the otic placode results in abnormal otic vesicle development. Intriguingly, *spalt4* appears to initiate a placode program appropriate for the axial level but is not involved in later development of specific placode fates. The results suggest that *spalt4* is involved in early stages of placode development, initiating ectodermal invagination and region-specific gene regulatory networks.

7. Ancient evolutionary origin of the neural crest gene regulatory network

Tatjana Sauka-Spengler, Daniel Meulemans, Matthew Jones, Marianne Bronner-Fraser

The neural crest is a uniquely vertebrate cell type that migrates from its origin at the embryonic neural plate border to form diverse derivatives including peripheral ganglia, craniofacial skeleton and melanocytes. Our previous analyses led to a hypothesis that a gene regulatory network, common to all vertebrates, guides formation of the neural crest. Here, we investigate when during evolution the hypothesized neural crest gene regulatory network (NC-GRN) emerged by experimentally analyzing the lamprey *(Petromyzon marinus)*, a basal extant vertebrate amenable to genetic and embryological

manipulations. Using an arrayed library, we identify 50 NC-GRN gene homologues and then use a morpholino-mediated loss-of-function approach to demonstrate a critical role for eight transcriptional regulators. The results reveal conservation of gene deployment for upstream transcription factors, suggesting that the proximal portion of this regulatory network arose early in vertebrate evolution and has been tightly conserved for over 500 million years. In contrast to this core conservation, we note differences in deployment of some specifier and downstream effector genes expected to confer species-specific migratory and differentiative properties. By testing the collective expression and function of a set of transcription factors within this putative gene regulatory network in a single vertebrate, the results reveal the basal ground state of the network and also help resolve ambiguities between model organisms.

Publications

Bailey, A.P., Bhattacharyya, S., Bronner-Fraser, M. and Streita, A. (2006) Lens specification is the ground state of all sensory placodes, from which FGF promotes olfactory identity. *Dev. Cell* **11**:505-517.

Barembaum, M. and Bronner-Fraser, M. (2007) Spalt4 mediates invagination and otic placode gene expression in cranial ectoderm. *Development* **134**:3805-3814.

Epperlein, H.H., Selleck, M.A.J., Meulemans, D., Mchedlishvili, L., Cerny, R., Sobkow, L. and Bronner-Fraser, M. (2007) Migratory patterns and developmental potential of trunk neural crest cells in the axolotl embryo. *Dev. Dyn.* **236**:389-403.

Gammill, L., Gonzales, C. and Bronner-Fraser, M. (2006) Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation. *J. Neurobiology* **67**:47-56.

Kee, Y., Hwang, B.-J., Sternberg, P.W. and Bronner-Fraser, M. (2007) Evolutionary conservation of cell migration genes: from nematode neurons to vertebrate neural crest. *Genes & Dev.* **15**:391-396.

Lwigale, P. and Bronner-Fraser M. (2007) Lens-derived Semaphorin3A regulates sensory innervation of the cornea. *Dev. Biol.* **15**:750-759.

McCauley, D. and Bronner-Fraser, M. (2006) Importance of SoxE in neural crest development and evolution of the pharynx. *Nature* **441**:750-752.

Meulemans, D. and Bronner-Fraser, M. (2007) A genetic model for the evolution of the vertebrate head skeleton. *PLoS One* 2, e787.

Sauka-Spengler, T., Meulemans, D. and Bronner-Fraser, M. (2007) Ancient evolutionary origin of the neural crest gene regulatory network. *Dev. Cell* **13**:405-420.

Taneyhill, L., Coles, E. and Bronner-Fraser, M. (2007) Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. *Developmen*t **134**:1481-1490.

Trinh, L, McCutchen, M., Bronner-Fraser, M., Fraser, S.E., Bumm, L.A. and McCauley, D.W. (2007) Fluorescent *in situ* hybridization employing the conventional NBT/BCIP chromogenic stain. *Biotechniques* **42**:756-759.

Yu, J.-K, Satou, Y., Holland, N.D., Shin-I, T., Kohara, Y., Satoh, N., Bronner-Fraser, M. and Holland, L.Z. (2007) Axial patterning in cephalochordates and the evolutionary origin of the gastrula organizer. *Nature* **445**:613-617.

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Summary: The major focus of research in our laboratory is on gene regulatory networks (GRNs) that control development, and the evolution of these networks. Most of our research is done on sea urchin embryos, which provide key experimental advantages. Among these are: an easy gene transfer technology, which makes this a system of choice for studying the genomic regulatory code; advanced technologies for high throughput perturbation of gene expression in the embryo; multiple means of visualizing and measuring gene expression; availability of embryonic material at all seasons of the year; an optically clear, easily handled embryo that is remarkably able to withstand micromanipulations, injections and blastomere recombination and disaggregation procedures; a very well understood and relatively simple embryonic process; and in-house egg-to-egg culture of the species we work with, *Strongylocentrotus purpuratus* (in a special culture system we have developed, located at Caltech's Kerckhoff Marine Laboratory). There is also a rich collection of arrayed cDNA and BAC libraries for many other species of sea urchins, at various degrees of relatedness to this one. The genome of *S. purpuratus* has been sequenced at HGSC (Baylor) and annotated. The experimental model that we utilize for evolutionary GRN comparisons is another echinoderm, also of local provenance, the starfish *Asterina miniata*. The embryo of this animal proves to be as excellent a subject for gene regulation molecular biology as is that of the sea urchin.

We pursue an integrated, "vertical" mode of experimental analysis, in that our experiments are directed at all levels of biological organization, extending from the transcription factor-DNA interactions that control spatial and temporal expression of specific genes to the system-level analysis of large regulatory networks. It has become apparent that the only level of analysis from which explanations of major developmental phenomena directly emerge, is the GRN system level.

The main research initiatives at the present time are as follows: ***i. Analysis of the gene regulatory network underlying endomesoderm specification in* S. purpuratus *embryos*:** At present about 50 genes have been linked into this GRN. The architecture of the network is emerging from an interdisciplinary approach in which computational analysis is applied to perturbation data obtained by gene expression knockouts and other methods, combined with experimental embryology. A predictive model of the GRN has emerged which indicates the inputs and outputs of the *cis*-regulatory elements at its key nodes. This model essentially provides the genomic regulatory code for specification of the endomesodermal territories of the embryo, up to gastrula stage. Most of the individual projects reported below are contributing to understanding of this network. ***ii. Testing the* cis*-regulatory predictions of the GRN:*** The GRN was constructed essentially by integrating the results of a massive perturbation analysis of expression of individual genes with spatial and temporal expression data for these genes. It predicts the required specific regulatory inputs and outputs linking the genes within the GRN. These predictions are subject to direct experimental *cis*-regulatory test, and correction. We have now authenticated the predicted *cis*-regulatory inputs into genes at a majority of the key nodes of the current GRN. At these nodes are regulatory genes into which there are multiple regulatory inputs from genes elsewhere in the

GRN, and multiple outputs to other genes in the GRN. For some regions of the GRN the analysis is approaching maturity, in that it extends convincingly from maternal inputs to cell-type differentiation. The best example is the GRN subregion determining skeletogenic micromere specification. Overall, the results of these experiments are converting the GRN from a model proposition into a hard-wired map of the genomic control logic for this portion of development. ***iii. Completion of the repertoire of regulatory genes engaged in the endomesoderm GRN:*** We used the data emerging from the genome sequence project to identify and assemble computationally all gene sequences that encode transcription factors. The temporal patterns of expression of these genes were determined, and for those genes sufficiently expressed in the embryo, the spatial patterns as well. Regulatory genes were identified in this manner that evidently play a role in endomesoderm specification, because they are expressed specifically in the endomesodermal territories at the relevant times, but that had not yet been incorporated into the GRN. All of these genes are now being linked into the GRN by perturbation and *cis*-regulatory analysis; this project has been completed for the skeletogenic micromere lineage and is in process for the non-skeletogenic mesoderm and the endoderm. ***iv. Evolution, viewed as a process of change in GRN architecture:*** Starfish and sea urchins shared a last common ancestor about 500 million years ago. Thus, analysis of the GRN controlling endomesoderm specification events in the starfish embryo will reveal both the nature of functional change in the GRN, and conservation of features that are so essential that they have resisted alteration for half a billion years. Examples of both have now been documented. The underlying processes are of course change, or alternatively, conservation, of functional *cis*-regulatory features. To study this we are examining starfish/sea urchin GRN differences at the *cis*-regulatory level. In a separate, large-scale effort, we have nearly completed the isolation of BACs containing 12 genes, the *cis*-regulatory elements of which are known in *S. purpuratus*, from genomic libraries of five different sea urchin species ranging from 15 to 250 million years since divergence from the lineage leading to *S. purpuratus*. These will afford the opportunity of studying by computational and experimental methods the process of *cis*-regulatory evolution, which is very poorly understood. We have also undertaken to determine how it occurred in the relatively recent evolution of sea urchins that an embryonic skeletogenic micromere lineage appeared, as this is the only echinoid class that displays such. The answer is that the whole adult skeletogenic control system was hijacked and targeted to the micromere lineage by a relatively small regulatory alteration. ***v. Oral and aboral ectoderm GRNs:*** We have begun work on the GRNs that control oral and aboral ectoderm specification. This is part of an effort to extend the same kind of causal, system level GRN analysis to the whole embryo. There is one additional early embryonic territory, the apical neurogenic region, which is being studied in other sea urchin laboratories. The aboral ectoderm generates a single cell type, but the oral ectoderm gives rise to several distinctly functioning domains: mouth, columnar "facial" epithelium, neurogenic ciliary band, and the ectodermal signaling stripes which determine the location of the skeletal rods. The approach is to obtain all the regulatory players expressed in oral and aboral ectoderm from the analysis of all genes encoding transcription factors predicted in the genomic sequence, and engage them in a provisional network by carrying out a matrix of perturbation experiments, at this writing close to completion. The network will be anchored at the onset of the ectodermal specification process, of which the initial gene zygotically expressed on the oral side is *nodal*. The *cis*-regulatory module controlling early oral ectoderm expression of nodal has been isolated and thoroughly characterized: its target sites provide the direct links between the initial cytoplasmic anisotropy by which the future oral and aboral sides of the embryo are distinguished, i.e., differences in redox potential, and differential zygotic gene expression. ***vi. Computational and experimental* cis-*regulatory model:*** To build a logic model of the information processing functions of a *cis*-regulatory element that relates the input kinetics (i.e., the temporal changes in relevant transcription factor levels) to its output, we returned to the *cyIIIa* gene. The logic model of the regulatory system of this gene was completed by additional mutational gene transfer experiments. ***vii. Various explorations by new methods and approaches:*** As always, we are trying to expand knowledge by use of novel technologies for analysis of the GRN and the genome. Current applications of new technology include increasingly widespread use of *in vitro* reengineered BAC recombinants, which we are supplying to the whole sea urchin field; use of these in first attempts to "redesign" the process of embryonic development, by introduction of altered regulatory subcircuits in novel spatial domains; and extensive application of two color gene transfer experiments in which the control version of a *cis*-regulatory construct drives expression of a reporter detectable in one color and a mutated version injected with it and incorporated in the same cells drives expression of a reporter detected in a different color. We have also developed a completely novel method for blocking expression of any gene whenever and wherever desired, though this has so far been tested only in sea urchin embryo skeletogenic cells. This method allows us to determine the function of regulatory genes that have multiple activity phases in one of the later phases, in embryos that develop normally up to that point. ***viii. Computational approaches to regulatory gene network analysis:*** The GRN visualization software BioTapestry, developed by our collaborators Hamid Bolouri and Wm. Longabaugh at ISB, is now in wide use, and we are further expanding its capacities so that it will automatically generate allowed network architectures from machine readable time and space of expression data plus results of perturbation analysis. A second-generation version with much enhanced capacities has been published. Many additional computational genomics and

other projects are summarized below. ***ix.*** An integrated treatment of genomic regulatory systems in development and evolution at both *cis*-regulatory and network levels is presented in a new treatise by the PI, "The Regulatory Genome" (Academic Press/Elsevier, 2006). This work contains a general treatment of developmental gene regulatory network theory and practice up to the present.

The Center for Computational Regulatory Genomics CCRG Genomics Technology Facility

R. Andrew Cameron, Director

Eric H. Davidson, Principal Investigator

The operation of the Facility centers on the Genetix Arraying Robot, a large flatbed robotic arm with video camera used to produce bacterial macro-array libraries and filters. We currently maintain in -80°C freezers 27 different echinoderm libraries comprising a total of approximately 3 million arrayed clones. In addition to providing these materials to academic research groups, we also offer the opportunity for outside groups from Caltech and elsewhere to array and spot their own libraries. During the past year we have arrayed a total 462,518 colonies and printed a total of 112 macroarray filters.

We continue to construct and array new libraries for use by the outside research community and our own work on gene regulatory networks including the evolution of *cis*-regulatory modules. The existing genomic DNA and cDNA libraries that were so extensively employed for the annotation of the sea urchin genome are stably maintained in our freezers. We print new filters for these as needed.

In response to a request from the Baylor College of Medicine, Human Genome Sequencing Center, we have prepared genomic DNA from two additional species, *Strongylocentrotus franciscanus* and the deep-water species, *Allocentrotus fragilis*. With the cooperation of Monterey Bay fisherman and colleagues at Hopkins Marine Station, Stanford University, Pacific Grove, California we were able to collect material for *Allocentrotus* DNA isolation and BAC library preparation. We have a BAC library and DNA from a single male *S. franciscanus* on hand.

Research Center

The goal of the Center for Computational Regulatory Biology is to develop, refine and test computational approaches in genomics broadly and *cis*-regulatory analysis specifically. The primary focus for the latter is the elucidation of gene regulatory networks in development. The Center interacts with the wider research community in several ways: it provides open source software for use by academic research groups; it provides web-based servers for genomic analysis using software developed locally; and it maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility. The Facility provides to the Caltech and external scientific community upon request services and materials stemming from the macroarray libraries and arraying equipment that we maintain.

One aspect of the Center is the Sea Urchin Genome Resource that maintains information resources that are used widely in the sea urchin research community. We provide sequence information through the Sea Urchin Genome Project web site (http://sugp.caltech.edu/). With the advent of the web resources for annotation established at the Human Genome Sequencing Center, Baylor College of Medicine and the Sea Urchin Genome Resources at NCBI, we have not seen the need to expand our local databases. However, we have refined the cross-index between our library clones and sequences stored in public databases at NCBI. Since so many of our libraries were used for the sequencing project, and the library location for the clones was preserved in the sequence information, we can provide a searchable sequence database from which the user can obtain clone information and order the clone. This "clone by computer" method renders our arrayed libraries extremely useful and readily accessible for the working molecular biologist.

As in previous years our sequence analysis software and server continue to be productively used. Sixty-one laboratories situated all over the US and Europe, and extending to Argentina, maintains folders on our server. Since the last startup in February 2005, there have been almost 13,000 Seqcomp program job requests accomplished for a total of about 1350 CPU hours. The largest number of request has been for Blast jobs but they have only used about 868 CPU hours.

Beowulf cluster hardware and configuration

Our 40-unit Beowulf cluster continues to operate with a minimum of downtime. The Rocks software stack of software components has indeed proven to be an efficient system with which to build, operate and maintain the cluster. We are processing the entire genome sequence on the cluster in various ways as part of the genome database. We further expect to queue search jobs from the genome database through this cluster.

8. The sea urchin genome annotation database and allied activities

R. Andrew Cameron, Kris Khamvongsa, Emmanuelle Morin

We are constructing a Sea Urchin Model Organism Database to preserve the unique new data emerging from the completion of the draft genome sequence for the purple sea urchin. The annotation data include, at last count, more than 10,000 individual records of manual curation based on gene models emerging from the work of the Sea Urchin Genome Sequencing Project. Much of these data are unique and not part of the sequence submissions to Genbank. They include corrected sequences, expression information and homology research the magnitude of which reflects the strong motivation of the annotation team. In addition, expression data from a whole genome tiling path microarray for embryonic RNA has been cross-indexed to the sequence data. Our primary

goal is to preserve this information in a database (Spbase) now that the primary annotation is finished and make sure it is immediately available to the community.

We have based the software for the site on the open source GMOD programs (http://gmod.org) sponsored by the NIH. We use the Chado database schema to structure a PostGres relational database complete with ontologies developed by GMOD that was constructed with input from a variety of highly successful genome databases. Through XML translaters, the database is connected to a sequence browser (Gbrowse) and a sequence annotator and editor (Apollo). The latest annotations (Freeze4) from Baylor are being processed into a format compatible with the GMOD software. Several different gene predictions are now mapped on top the genome assembly scaffolds along with the gene names themselves. At present we have a consistent name list for more that 9,000 genes form the annotation project.

9. The sea urchin repeat sequence complement

Susan Ernst[*], *Emmanuelle Morin, R. Andrew Cameron*

Repeat sequences make up a major portion of animal genomes, about 55% in humans and 35% in the purple sea urchin. Sea urchin repeat sequences were well studied by solution hybridization about 30 years ago. We mounted a group of software packages to create a pipeline for analyzing the repeats from the latest sea urchin genome assembly (Spur v2.1). They include RECON, WUBlast, and Dialign among them. The basic strategy is to iterate through an all-by-all blast analysis followed by repeat clustering and genome sequence masking on ever increasing portions of the genome. We have completed analysis of 7500 randomly chosen 20 Kb sequences amounting to 18% of the genome. We should have captured any repeat randomly present in the genome more that 10 times. At this point, we are reaching an overlap with duplicated gene families and statistical noise given the draft level of the genome sequence. We have begun to annotate those repeat families that are present in our sample 10 times (or 50 times in the genome). Not unexpectedly, we are finding good representatives of both LTR and non-LTR retrotransposons and a rich complement of short interspersed nuclear elements or SINES is also detected.

[*]*Tufts University, Medford, MA*

10. Toward a gene chip for embryonic gene expression studies

Eve Helguero, Manoj Samanta[*], *R. Andrew Cameron*

A whole genome-tiling array was used to measure the expression of all genes regulated during early embryogenesis. This proved to be invaluable during the annotation of the predicted gene models in the sea urchin genome. Using these data and the predicted sequences we have designed a new array of tiles that covers just the genes expressed during embryogenesis and the large suite of innate immune genes, some of which are expressed during embryogenesis. This new array has been constructed by Nimblegen and sent to the Millard and Muriel Jacobs Genetics and Genomics Laboratory in the Division of Biology. Preliminary testing of this array with newly prepared RNA populations identical to the ones used on the genome array is producing surprisingly similar results in both in terms of level and in comparison to other genes in the population. We continue test the array for eventual use as a tool in the measurement of gene expression during embryonic perturbations, a mainstay of our gene regulatory network analyses.

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11. Network gene annotation project

Qiu Autumn Yuan, R. Andrew Cameron

We had previously built and continue to maintain a database of sequence and expression data for about 550 sea urchin regulatory genes. The data housed in this suite of databases continues to grow as additional genes, expression patterns and network linkages are added to the existing information. These unpublished data ate available to a registered user group through an unlinked Caltech web site. For each gene in the database the information includes fields with the annotations of the gene, its various sequence objects and expression data. This database has been newly implemented in PostgreSQL. The functions of the website and management of the database have been scripted in a variety of languages: HTML, XML, CSS, PHP and Javascript. As these data are codified, they will be used to directly erect gene regulatory networks in a computational version of BioTapestry, our GRN viewer and editor.

Our client base has grown considerably over the past year and new users have added more biological data. We have also added to the suite a new database that organizes the sequences from the whole-genome tiling path according to the genes they contain. Because of the strategy used to sequence the genome, each scaffold sequence is aligned to a BAC that originated in our libraries. Thus, one can obtain a genomic sequence clone directly or "clone by computer." We have had numerous requests for these data and we expect that a searchable database will be very useful.

12. The evolution of *cis*-regulatory module sequence in lower deuterostomes

R. Andrew Cameron, Elly Chow, Eve Helguero, Qiu Autumn Yuan, Ping Dong, Julie Hahn, Rena Schweizer[*]

We have made comparisons of functionally characterized *cis*-regulatory modules from the *Strongylocentrotus purpuratus* genome, and the orthologous regulatory and flanking sequences from a BAC genome library of a congener, *Strongylocentrotus franciscanus*, which are about 20 million years diverged from each other. These studies reveal that single nucleotide substitutions and small indels occur freely at many positions within the regulatory modules of these two species, as they do without. However, large indels (>20

bp) are statistically almost absent within, though they are common in flanking intergenic or intronic sequence.

We have added two new comparisons between *Strongylocentrotus purpuratus* and *S. fransciscanus* to the analysis discussed above. Furthermore we have expanded the panel of comparisons to be made. We now have partial results spanning five species and ten genes. Recombinant BAC clones in which GFP is substituted for the gene-coding region while the surrounding genomic sequence is preserved have proved efficient constructs to test the regulatory function of large DNA segments. Constructs were obtained for six of the 10 genes in our panel from *Arbacia punctulata* (Ap), a sea urchin diverged from the reference species for about 150 MY.

We have provided DNA from *Strongylocentotus franciscanus* and *Allocentrotus fragilis* to the Baylor College of Medicine Human Genome Sequencing Center. The Baylor HGSC wishes to use these genomic DNAs to test the feasibility of sequencing large animal genomes on the new 454 sequencing platform and use the purple sea urchin genome sequence to aid in the assembly process. Preliminary results from this enterprise are quite favorable. About 2 million reads from the ~820 Mb genome of *S. franciscanus* have been produced and about 550 thousand reads have been accumulated from the *A. fragilis* genome. More than 80% of the reads in both genomes can be mapped to the reference species sequence. We are currently analyzing these sequences in order to expand the use of the Indel Exclusion Rule for locating putative *cis*-regulatory modules computationally on a genome-wide basis.

[*] *UCLA*

13. Transcriptional control of the sea urchin *brachyury* gene

Elly Chow, R. Andrew Cameron

brachyury is expressed in a ring of cells that surround the presumptive mesoderm in the vegetal plate of the early sea urchin embryo and an intronic element controls this expression. Our examination of the vegetal plate regulation of *brachyury* has matured in the last year. We are certain that the major effectors of expression in this territory are Tcf and Gatae. *brachyury* is activated by the Tcf transcription factor and its cofactor, β-catenin. In the absence of β-catenin, Tcf combines with the ubiquitous repressor, *groucho* to prevent *brachyury* expression in the ectoderm and the mesoderm territories. Gatae is another activator of *brachyury* that is expressed only in the vegetal plate. The dynamic expression of *brachyury* which involves progressive inactivation of expression in the cells that invaginate to form the archenteron is likely to involve the Tcf system, however other possible regulators in the advancing endoderm have not been identified. A third activator is implied because the intronic element with all of the Tcf sites mutated is expressed ectopically in the ectoderm. We hypothesize that this could be an Ets factor because several canonical Ets factor binding sites are found in the sequence and two Ets factors, Sp-Erf and Sp-ElfA, are ubiquitously expressed at the right time. We are currently testing this idea with constructs in which all of the Tcf and Gatae sites are mutated.

14. A gene regulatory network subcircuit that drives a dynamic pattern of gene expression

Joel Smith, Christina Theodoris, Hongdau Liu[*]

Early specification of endomesodermal territories in the sea urchin embryo depends on a dynamically moving torus of regulatory gene expression. Expression begins at the vegetal pole of the cleavage-stage embryo and moves outward, encompassing successive concentric tiers of future mesoderm and endoderm cells. Meanwhile the same regulatory genes are progressively repressed in an expanding disc of cells in the center of the vegetal plate, generating the moving torus pattern. Here we show how this dynamic patterning function is encoded in a gene regulatory network subcircuit. The key components of the subcircuit are the *otx*, *wnt8* and *blimp1b* genes, the *cis*-regulatory control systems of which have all been experimentally defined. A *cis*-regulatory reconstruction experiment directly demonstrates that *blimp1b* autorepression accounts for progressive extinction of expression in the center of the torus, while its outward expansion follows reception of the Wnt8 ligand by adjacent cells.

[*] *Undergraduate, Caltech*

15. A spatially dynamic cohort of regulatory genes in the endomesodermal gene network of the sea urchin embryo

Joel Smith, Ebba Kraemer, Hongdau Liu[*], *Christina Theodoris*

A gene regulatory network subcircuit comprising the *otx*, *wnt8* and *blimp1b* genes accounts for a moving torus of gene expression that sweeps concentrically across the vegetal domain of the sea urchin embryo. Here we confirm by mutation the inputs into the *blimp1b* *cis*-regulatory module predicted by network analysis. Its essential design feature is that it includes both activation and autorepression sites. The *wnt8* gene is functionally linked into the subcircuit in that cells receiving this ligand generate a β-catenin/Tcf input required for *blimp1b* expression, while the *wnt8* gene in turn requires a Blimp1 input. Spatial expression patterns and gene regulatory analysis indicate the genes *even-skipped* and *hox11/13b* are also entrained by this subcircuit. We verify the *cis*-regulatory inputs of *even-skipped* predicted by network analysis. These include activation by β-catenin/Tcf and Blimp1, and repression by Hox11/13b. Thus, *even-skipped* and *hox11/13b*, along with *blimp1b* and *wnt8*, are members of a cohort of torus genes with similar regulatory inputs and similar, though slightly out-of-phase, expression patterns, which reflect differences in *cis*-regulatory design.

[*] *Undergraduate, Caltech*

16. Developmental stage- and tissue-specific gene knockdown in the sea urchin

Joel Smith

Though the use of morpholino-substituted antisense oligonucleotides has proven immeasurably useful for determining the sea urchin gene regulatory network for endomesoderm specification, this method of gene knockdown is limited in that the effect of downregulation decays with time and in any case may not be appropriate if target genes have additional, essential early roles. We report a technology to sidestep these problems by regulating the spatial and temporal effects of targeted gene knockdown. This method uses DNA expression constructs where time- and tissue-specific *cis*-regulatory sequences drive transcription of splice-blocking antisense oligomers. We use this method to achieve spatially- and temporally-restricted knockdown of two genes, Ets1 and Alx1, critical for proper skeletogenesis in the purple sea urchin, *Strongylocentrotus purpuratus*. Using early-acting enhancer sequences derived from the promoter for the Tbr gene in order to drive expression specifically in the skeletogenic cells, we target either Ets1 or Alx1 transcripts for destruction prior to the ingression of skeletogenic cells. We find this perturbation successfully blocks normal migration patterns as observed in control embryos. Using instead a late-acting enhancer from the Sm30 gene, which only runs in skeletogenic cells after ingression, we see normal cell ingression. Critically, these same test embryos show defects in later skeletogenesis activities. This reflects the downregulation of either Alx1 or Ets1 only after migration has occurred and when Sm30 promoter elements are activated, thereby demonstrating the strict control of antisense transgene expression. For this method no in-depth knowledge of promoter sequences is required, only basic means of transgenesis. This technique for achieving spatial/temporal control of gene knockdown should, therefore, have wide applications in virtually any system.

17. *cis*-Regulatory control of the *nodal* gene, initiator of the sea urchin oral ectoderm gene network

Jongmin Nam, Yi-Hsien Su, Pei Yun Lee

Expression of the *nodal* gene initiates the gene regulatory network that establishes the transcriptional specification of the oral ectoderm in the sea urchin embryo. This gene encodes a TGFβ ligand, and in *Strongylocentrotus purpuratus* its transcription is activated in the presumptive oral ectoderm at about the 30-cell stage. Thereafter, Nodal signaling occurs among all cells of the oral ectoderm territory, and nodal expression is required for expression of oral ectoderm regulatory genes. The *cis*-regulatory system of the *nodal* gene transduces anisotropically distributed cytoplasmic cues that distinguish the future oral and aboral domains of the early embryo. Here we establish the genomic basis for the initiation and maintenance of *nodal* gene expression in the oral ectoderm. Functional *cis*-regulatory control modules of the *nodal* gene were identified by interspecific sequence conservation. A 5' *cis*-regulatory module functions both to initiate expression of the *nodal* gene and to maintain its expression by means of feedback input from the Nodal signal transduction system. These functions are mediated respectively by target sites for bZIP transcription factors, and by SMAD target sites. At least one SMAD site is also needed for the initiation of expression. An intron module also contains SMAD sites that respond to Nodal feedback, and in addition acts to repress vegetal expression. These observations explain the main features of *nodal* expression in the oral ectoderm: since the activity of bZIP factors is redox sensitive, and the initial polarization of oral vs. aboral fate is manifested in a redox differential, the bZIP sites account for the activation of nodal on the oral side; and since the immediate early signal transduction response factors for Nodal are SMAD factors, the SMAD sites account for the feedback maintenance of nodal gene expression.

18. The subnetwork underlying specification of mesoderm in sea urchins

Stefan C. Materna, Gilson J. Sanchez[*]

Our current research aims at revealing the logic interactions between regulatory genes that lead to the specification of the mesoderm in the early sea urchin embryo. The vegetal half of the sea urchin embryo will give rise to mesodermal cell types that includes pigment cells, blastocoelar cells, coelomic pouch cell and esophageal muscle cells. At blastula stage, the progenitors cells of endoderm and mesoderm – the endomesoderm - form a concentric ring surrounding the cells of the skeletogenic lineage at the center of the vegetal plate. During 8^{th} cleavage this tier is subdivided radially into two rings, the inner of which will give rise to the mesoderm, the outer the endoderm.

Initially, the processes that lead to specification of endoderm and mesoderm are set in motion by the nuclearization of beta-catenin, which is part of a re-enforcing, regulatory circuit that involves the *blimp1/krox* and the *wnt8* gene. This subcircuit is first active in the cells of the skeletogenic lineage and then moves in a torus like motion across the vegetal half of the embryo. In endomesodermal cells it activates genes that are essential for their specification, e.g., the *otx* gene. Following the subdivision of endomesoderm into endodermal and mesodermal cells, a Delta signal is expressed by the skeletogenic cells and received by the mesodermal cells via the Notch receptor. This signal is the decisive clue that causes the mesoderm to adopt a different fate from endoderm. After reception of this signal, some strictly mesodermal genes are activated, including the *glial cells missing* gene. This gene has been shown to be a direct downstream target of Notch signaling. It is responsible for the specification of pigment cells and activates markers, as the *polyketide synthase* gene, which are specific to pigment cells.

The above description is only a rough sketch of the processes known to be important in mesoderm development. Despite accumulating knowledge of the

subcircuitry, several questions remain unanswered. For example, it is unclear how genes activated by Blimp1/Wnt8/Otx relate to those activated by Delta/Notch signaling. In order to close the existing knowledge gaps in the mesodermal subnetwork, we are currently in the process of integrating new candidate genes, which have been identified as part of recent efforts to identify all the transcription factors in the sea urchin genome, into the existing network. This will improve our understanding of the logic operations that need to be executed in order to specify mesoderm in the sea urchin embryo.

Cal State, LA.

19. *Trans*-specification of primary mesenchyme cells through genetic rewiring of the mesoderm specification network

Sagar Damle

In the sea urchin *Strongylcentrotus purpuratus*, the identity and regulatory relationship of a number of transcription factors involved in endomesoderm development have been well characterized. However, the ultimate demonstration of intellectual control of the causal moving parts of a system is to reengineer it. The goal of the project is to determine whether the ectopic expression of the transcription factor *SpGcm* is sufficient to trans-specify primary mesenchyme cells (PMC) into a secondary mesenchyme cell (SMC) fate. This can be done by placing the *gcm* coding sequence under the control of a promoter that directs PMC-specific gene expression.

To this end, the Davidson lab has developed a protocol whereby BAC-sized genomic DNA reporter constructs can be introduced into fertilized sea urchin eggs through microinjection and integrated into the genome as early as the two-cell stage. BAC based expression constructs, being typically between 50 and 150 kb in size, have a distinct advantage over smaller constructs in that they may contain the full *cis*-regulatory system responsible for controlling the expression of a gene. As such, reporter genes driven by these BACs can accurately recapitulate endogenous gene expression both spatially and temporally during development. When used to drive a transgene, as is proposed here, these BAC constructs offer a method for respecification of cell types.

SpGcm is thought to play two roles in development. Its zygotic expression begins during mid-blastula stage. Delta signaling by PMC cells induces *SpGcm* expression within the veg2 tier of cells, initially only in the layer of cells immediately adjacent to PMCs, and later in neighboring veg2 cells. During gastrulation, these SMCs continue to express *SpGcm*. It accumulates predominantly in pigment cells that slough off the aboral tip of the archenteron and migrate to the aboral ectoderm. Its early expression in this region of veg2 endomesoderm marks all presumptive secondary mesenchyme lineages and suggests that, given the appropriate transcriptional context, it may be capable of setting up a mesodermal cell fate. This state gives cells a competency to respond to signals that specify various SMC or mesodermal cell lineages. Some evidence for this already exists. For instance, embryos injected with antisense morpholino against *gcm* do not correctly express the *gata-c* gene in the oral domain of veg2 mesoderm (A. Ransick and J. Rast, unpublished data). Morpholino-injected embryos also fail to form pigment cells.

PMCs, which are also mesodermal in origin and differentiate into skeletogenic cells, maintain a transcriptional state that may be very similar to that of SMCs. Indeed, SMCs are capable of autonomous respecification into PMCs in PMC-depleted larvae. A number of transcription factors necessary for PMC skeletogenesis and ingression into the blastocoel. Of these, the T-box transcription factor Tbrain is expressed exclusively in the large micromere descendants at swimming blastula stage. Its expression persists through gastrulation into PMCs. A recombinant BAC has been constructed whereby *gcm*-coding sequence is transcribed under the control of the *tbrain cis*-regulatory system. This transgene system has been used to induce PMC-specific expression of *gcm* during blastula and gastrula stages. Whole-mount *in situ* hybridization (WMISH) of blastula-stage embryos have shown that transgenic PMCs induce expression of differentiation genes of the pigment cell lineage, including *SpPks* and *SpSult*. They also show a loss of several transcription factors necessary for PMC-specification or skeletogenesis including *SpAlx*, *Spfoxb* and *SpJun* as well as a differentiation gene *SpMsp130*. Through the use of an Alx-GFP BAC reporter it has become possible to monitor Alx transcription in converted cells. qPCR experiments performed on embryos coinjected with a Tbrain-GCM BAC and an Alx-GFP reporter show a similar downregulation of Alx at mesenchyme blastula stage. Consistent with these observations, *Gcm*-expressing PMCs do not appear to participate in skeletogenesis. Their ingression is delayed, compared to normal PMCs and when they do ingress, some migrate towards the aboral ectoderm in a manner similar to their pigment cell relatives.

To obtain quantitative data on gene regulatory effects, we have performed qPCR on sorted PMCs coexpressing both GCM and a reporter GFP (both under control of Tbrain *cis*-regulatory DNA). Preliminary results of these experiments correlate with WMISH data, however both suggest that *Gcm*'s effect on SMC specification may be dosage dependent. To this end, methods of amplifying GCM expression in PMCs are being explored.

20. *cis*-Regulatory analysis of *Sptbr*, a key node in the micromere/PMC regulatory network

Mary Wahl, Paola Oliveri

The sea urchin endomesoderm gene regulatory network (GRN) is a model that describes the genetic program controlling the early stages of specification and development in *Strongylocentrotus purpuratus.* The *tbr* gene is a key node of the micromere/primary mesenchyme cell (PMC) sub-network. Zygotic *tbr* expression responds to the double repressive inputs downstream of *pmar1* and *hesC*, and its appearance is exclusive to the micromere lineage, (similar to *delta*, *ets1* and *alx1*). The

cis-regulatory analysis of *tbr* is essential for validating the inputs modeled in the endomesoderm GRN and to understand the regulatory code for this developmental event.

The annotated *Sptbr* BAC clone shows that *tbr* lies on a gene-dense fragment of DNA. The BAC contains six genes in ~130 kb and *tbr* is flanked by genes 3.5 kb upstream and 16 kb downstream.

A *SpTbr* GFP BAC knock-in construct recapitulates the endogenous expression pattern and responds to the *pmar1* and *hesC* perturbation, showing that the large BAC contains all the regulatory elements necessary for correct expression.

By deletion analysis we identified a small 343 bp fragment, called γ(2) and located upstream of the coding sequence. γ(2) is sufficient to drive the expression of the reporter gene correctly in PMC lineage and responds accurately to the perturbation of the double repressive inputs.

In order to identify any putative transcription factor binding sites, we performed electrophoretic mobility shift assays (EMSA) using 12 hr nuclear extract and a set of overlapping oligos (30 bp) covering the entire γ(2) region. We also performed specific competitions to better redefine the sequence bound by transcription factors present in the nuclear extract. This result shows 11 specific DNA-protein complexes. Some of them if mutated or deleted have no effect on the spatial regulation of the reporter gene. For instance, when the single Otx site as well as complex 2, 4 and 6 are mutated, the construct still drives the expression of the reporter gene in the correct cell type. On the contrary, two binding sites for a potential Ets factor and a site for a bHLH factor appear to be critical for the expression of the reporter gene. Roger Revilla has reported a similar situation for the PMC-specific module of *delta* gene (C1 of R11), in which the Ets binding sites are essential for the expression of the reporter gene. In the *tbr* and *delta* *cis*-regulatory apparatuses an Ets factor might be responsible for the ubiquitous positive inputs and HesC, through a non-canonical site or by direct binding to the Ets factor, would be responsible for the repression in every cell except for the micromere/PMC lineage.

Further characterization of the regulatory code for early micromere/PMC expression, the nature of the transcription factors, and the molecular mechanism of HesC repression are under investigation.

21. *foxA* *cis*-regulatory analysis at early development in the sea urchin embryo

Smadar Ben-Tabou de-Leon

foxA is one of the key regulatory genes in the gene regulatory network governing endomesoderm development in sea urchin. It is necessary for the endoderm specification, for gut formation and for exclusion of mesodermal fate in endodermal cells. *foxa* is initially expressed in both the mesoderm and the endoderm territories, but a few hours after its activation it is shut off in the mesoderm and remains only in the endoderm. At 24 hr after fertilization *foxa* is expressed in the oral ectoderm and is necessary formation of the mouth. *foxa* activates the expression of the Hedgehog ligand and represses the expression of the key mesoderm specification gene, *gcm*, in the endoderm. *foxa* down regulates its own expression and accordingly its level oscillates with time. Understanding the regulation of the *foxa* dynamic expression will shed light on the endoderm fate decision and will enable a thorough analysis of the kinetics of an auto repressor.

GFP and RFP knock-in into *foxa* BAC were used to identify the *cis*-regulatory element that controls the *foxa* expression up to 72 hr after fertilization. The *cis*-regulatory element contains a 1 kb proximal module laying immediately 5' upstream of the *foxa* start of transcription, and a distal 1.3 kb module laying 10 kb 5' upstream. The proximal module gives rise to ubiquitous expression and the distal module restrains the expression to the correct endoderm and oral ectoderm pattern. In agreement with the gene regulatory network prediction for the *foxa* inputs, the *cis*-regulatory element contains multiple *gatae* and *otx* binding sites. The distal module contains five *foxa* binding sites that could correspond to the *foxa* auto repression. The distal module contains three Tcf1 binding sites that could explain the restriction of the expression of *foxa* to the endoderm. The function of these putative binding sites will be experimentally tested by mutation. This analysis will provide a complete model of the spatial and temporal expression pattern of the *foxa* gene and illuminate the endoderm lineage fate decision.

22. Re-engineering the ectoderm into endomesoderm by rewiring the sea urchin gene regulatory network

Smadar Ben-Tabou de-Leon, Lauren Lee, Andrew Ransick

The increasing understanding of the gene regulatory network that governs endomesoderm development in the sea urchin embryo opens the way to novel reengineering approach. This means that the key regulatory genes that initiate one domain specification can be turned on in another domain and change its fate. If the domain fate remains unaltered or changes only partially it is either that a necessary factor for respecification is missing or that the endogenous fate was already established so the domain lost its plasticity.

The animal half of the sea urchin embryo is specified into ectoderm through development. Transplantation of the most vegetal cells of the embryo, the micromeres, into the animal plate of another embryo induces respecification of the animal plate into endomesoderm lineage and to second gut formation. The transcription factor *pmar1* is expressed early in the micromeres and was shown to be upstream of the micromere specification program and of the endomesoderm induction signal. Activating *pmar1* early enough in the ectoderm should result in respecification of the cells into micromeres and induction of endomesoderm fate to the neighboring cells. In order to rewire the gene

regulatory network, we used the *cis*-regulatory module of the *nodal* gene to drive *pmar1* (Nodal:Pmar1) early in the oral ectoderm. Injection of the Nodal:Pmar1 construct to sea urchin eggs results with the respecification of oral ectoderm cells into micromere lineage cells that fuse with the endogenous micromeres to form a skeleton. However, no endomesoderm phenotypes in the oral ectoderm were observed. Previous studies had shown that *pmar1* expression in the ectoderm is sufficient to drive the gene *endo16*, an endoderm marker. These results show that Pmar1 expressing ectoderm cells are transformed to micromere lineage fate, but are able to drive only partial respecification of their neighboring cells. We will study the change in the regulatory genes expression in both Nodal:Pmar1 injected embryos and micromere-transplanted embryos by WMISH. This will illuminate the molecular differences underlying the phenotypic differences between these two experiments. It will also enable the identification of either the missing factors that are required for complete respecification or the lost of plasticity of the animal plate.

23. The gene regulatory network for ectoderm specification in the sea urchin embryos

Yi-Hsien Su

Although the gene regulatory network for oral-aboral ectoderm specification in the sea urchin embryo is yet undetermined, Nodal, a member of the TGF-b superfamily, is an early signaling molecule known to specify ectoderm formation. With the discovery from a genome-wide survey of thirty transcription factors that are expressed in the ectoderm, perturbation of Nodal expression by injection of morpholino followed by QPCR analysis shows that three oral transcription factors (foxG, gsc, and nk1), four aboral transcription factors (tbx2/3, irxA, nk2.2, and dlx), and three signaling molecules (Lefty, Chordin, and Bmp2/4) are regulated by Nodal. A preliminary oral-aboral ectoderm network emerges from the perturbation analyses of most of the thirty genes in conjunction with spatial expression information. The oral-aboral ectoderm network contains the basic characteristics of gene regulatory networks. The positive autoregulatory loops of nodal, foxG, gsc, and lim1, lock down the oral ectoderm cell fate. Two negative autoregulation loops (foxJ1 and nk2.2) are also observed. This emerging ectoderm network is a foundation for understanding the specification of oral-aboral ectoderm during early development of the sea urchin embryo.

24. Spatial expression patterns of genes in the oral/aboral domain of the purple sea urchin until 30 hours post fertilization

Alexander Kraemer

The spatial expression patterns of regulatory genes and transcription factors in the oral and aboral domain of the purple sea urchin *Strongylocentrotus purpuratus* have been studied. The purpose was to characterize spatial expression data and interpret the results in context of the current ectodermal Gene Regulatory Network (GRN) model. The gene expression patterns studied are: *nfe2, tbx2.3, Splim1, soxC, Spnk2.2, atbf, smadIP, e2f3, Spshr2, irxA, ecr, nk1, lhx2.9, hlf, tead4, dlx, mitf, hes, SpBMP2-4, chordin, lefty.* These genes represent a subset of a library of more than 600 known regulatory factors that had some activity in the oral and/or aboral domain. At relevant time points, RNA was extracted for cDNA synthesis and high resolution QPCR analysis revealed the hourly time course of expression of each gene in question throughout embryogenesis. Also anti-sense RNA probes were designed for the use in whole-mount *in situ* hybridization (WMISH). A time series (up to 30 hours post fertilization) of WMISH experiments at different developmental stages revealed the spatial expression pattern throughout the development. For those early developmental stages in which the sea urchin embryo is almost a symmetrical sphere and expression domains therefore cannot be specified with certainty based on the morphology of the embryo, a double WMISH was done for ten genes with respect to *spec1*, a gene known to have aboral expression. The spatial expression data obtained are stored in a database that holds all relevant gene information as well as the image libraries and an abstracted description of the expression patterns over time.

25. Deciphering the endoderm gene regulatory network

Isabelle Peter

The current model of the sea urchin endoderm gene regulatory network consists of twelve transcription factor genes and it predicts the regulatory logic by which they control the expression of each other. The genome-wide study of transcription factor gene expression patterns has revealed an additional set of eleven transcription factors that are expressed in the presumptive endoderm cells in the time period of specification. It is our aim to determine the regulatory relationships of all these transcription factors and to reconstruct the gene regulatory network driving endoderm development.

For each of the transcription factors it will be determined whether interfering with its expression affects the expression of other network components. In order to knock down the expression of regulatory genes, sea urchin embryos are injected with morpholino antisense substituted oligonucleotides (MASO) that specifically interfere with either translation or splicing of the targeted gene. Expression levels of all the genes in the endoderm network are being analyzed by QPCR in perturbed and control embryos and genes with significantly altered expression levels will be considered as potential target genes. These experiments will lead to the prediction of the regulatory inputs that control the expression of each gene in the network, which includes a set of endoderm differentiation genes and genes encoding signaling molecules in addition to the regulatory genes. Perturbation experiments have been performed for some of the transcription factors and future experiments will extend

this analysis to the whole set of endoderm specification genes.

26. A full dimension gene regulatory network for cell specification

Qiang Tu, Paola Oliveri, Jina Yun

Development of the body plan is controlled by large gene regulatory networks (GRNs). Here we report a GRN that directs the specification of primary mesenchyme cells (PMCs) in sea urchin embryos. The vast majority, if not all, regulatory genes involved in this developmental process were identified by comprehensive gene expression profiling. The casual relationships among them were elucidated by large-scale gene perturbation and *cis*-regulatory analyses. The architecture reveals that various network motifs are used to execute certain functions, including turning on by inherited anisotropy, development stage lock down by positive feedback loops, alternative fate exclusion by repression, and transient regulatory state dying out by auto repression. The model is able to explain all the developmental functions that PMCs execute, which are being initiated by the inherited anisotropy, emitting signals to adjacent cells, and initiating skeletogenic mesenchyme. This PMC GRN is a subnetwork of the larger GRN which directs the specification of endomesoderm in sea urchin embryos, and it is the first full-dimension GRN, meaning the vast majority of nodes and most linkages have been identified, which directs the specification of this particular cell type.

27. *cis*-Regulation of *Spgcm*: Late module functionalities

Andrew Ransick

A variety of experimental approaches are being employed to define the *cis*-regulatory architecture and critical 'trans' inputs of *Spgcm*, the sea urchin (*S. purpuratus*) ortholog of the *Drosophila* transcription factor *glial cells missing*. Four *cis*-regulatory modules of the *spgcm* gene have been identified, distributed across ~15 kilobases of sequence upstream of transcription start site. When incorporated in a GFP expression construct they recapitulate both the early expression pattern of this gene in the secondary mesenchyme cell (SMC) domain at the mesenchyme blastula (MB) stage, and the late expression domain in pigment cells (PC) of post-gastrula stage embryos and larvae. [For additional details, see Ransick and Davidson (2006) *Dev. Biol.* **297**:587-602]. The regulatory module controlling the late expression in PC has been the recent focal point. This so-called "G module" (spanning ~500 base stretch located ~4 kilobases upstream of the transcription start site) is composed of a clustered set of short conserved sequence elements. An auto-regulatory site in the form of a consensus site match to the canonical GCM binding site has been demonstrated to maintain *Spgcm* expression levels beginning around MB stage – providing a 3-4 fold boost in *Spgcm* mRNA levels. Additionally, *cis*-perturbation analyses have demonstrated that a 10 bp element comprising a consensus NFkB binding site is essential for driving *Spgcm* expression in PC from the time their precursors ingress at the start of gastrulation. Effort is now shifting toward characterization of the element(s) that mediates a dramatic down regulation of *Spgcm* on the oral side of the SMC domain in the early MB. As previous GRN experiments indicated that manifestation of this *Spgcm* functionality requires oral ectoderm (OE) specification, we expect this aspect of *Spgcm cis*-regulation provides a key inter-territory node in the GRN architecture that mediates second axis information from the OE territory to the SMC domain.

28. Evolution and rewiring of echinoderm skeletogenesis GRN

Feng Gao

All echinoderms make some form of calcareous skeleton as adults. However, only the class *Echinoidea* acquires an embryonic skeleton made by a precociously segregating micromere lineage dedicated to this task. Phylogeny and paleontology indicate the introduction of this new morphological character occurred since the P/T extinction 250 mya, the period after which the first euechinoids appeared. Knowledge of the gene regulatory network (GRN) allows us to think mechanistically about how evolutionary changes might occur, since changes in complex traits that require developmental processes must be reflected by changes in GRN architecture.

The micromere subnetwork of the sea urchin endomesoderm GRN consists of 31 genes presently. How the micromere lineage acquires its skeletogenic capability during embryogenesis is largely understood. To better understand adult skeletogenesis, we used the micromere GRN to select 23 candidate skeletogenic genes, and measured their expression patterns in the larval rudiment where the adult skeleton is made, and then mapped the results on the network architecture.

We found both embryonic and adult skeletons contain the products of differentiation genes like Sm30, Sm50 and MSP130. The main interesting result is the whole skeletogenic regulatory apparatus that runs in the embryo to make embryonic skeleton is evidently also employed in the larvae to make adult skeleton. This includes the skeletogenic regulatory state initiation genes like *alx1* and *ets1*; signaling genes like VRGFR; lockdown genes like *hex*, *tgif* and *erg*; and the immediate differentiation drivers like Dri. Therefore the gene batteries and the whole skeletogenic regulatory system of the micromeres, including its many elegant cross-regulatory interactions, was probably incorporated into micromeres in echinoderm evolution since the P/T extinction. The fusion point of this regulatory hijacking of a large gene network is the double negative Pmar1-HesC gate on the micromere GRN. This amazingly simple way of putting the whole control system into the micromere developmental address may illuminate why the hijacking could happen during evolution. What is excluded from the shared set are the micromere-specific regulatory genes *nβ-tcf*, *blimp1/krox* and *wnt8*; the micromere signaling genes ES, Delta and FoxN2/3; and some transcription factors like *tbr*, *tel* and *foxB*.

We also checked some genes from this skeletogenic network to investigate their use in the skeletogenesis of starfish, which shared a common ancestor with sea urchin about 500 My. Preliminary data indicates this network was in place before the divergence of sea urchin and starfish. Since we know the skeletogenic network of Echinoderm, our next goal is to make a starfish with embryonic skeleton through GRN rewiring.

Publications

Arnone, M.I., Rizzo, F., Annunciata, R., Cameron, R.A., Peterson, K.J. and Martinez, P. (2006) Genetic organization and embryonic expression of the ParaHox genes in the sea urchin *S. purpuratus*: Insights into the relationship between clustering and colinearity. *Dev. Biol*. **300**:63-73.

Ben-Tabou de-Leon, S. and Davidson, E.H. (2006) Deciphering the underlying mechanism of specification and differentiation: The sea urchin gene regulatory network. *Science* STKE (361), pe47. [DOI: 10.1126/stke.3612006pe47].

Ben-Tabou de-Leon, S. and Davidson, E.H. (2007) Gene regulation: Gene control network in development. *Ann. Rev. Biophys. Biomol. Struct*. **36**:191-212.

Bottjer, D.J., Davidson, E.H., Peterson, K.J. and Cameron, R.A. (2006) Paleogenomics of echinoderms. *Science* **314**:956-960.

Damle, S., Hanser, B., Davidson, E.H. and Fraser, S.E. (2006) Confocal quantification of *cis*-regulatory reporter gene expression in living sea urchin. *Dev. Biol.* **299**:543-550.

Davidson, E.H. (2006) Special issue: The sea urchin genome. *Dev. Biol*. **300**:1.

Davidson, E.H. (2006) The sea urchin genome: Where will it lead us? *Science* **314**:939-940.

Hinman, V.F., Nguyen, A. and Davidson, E.H. Caught in the evolutionary act: Precise *cis*-regulatory basis of difference in the organization of gene networks of sea stars and sea urchins. *Dev. Biol.* In press. Corrected proof available online 16 September, 2007.

Hinman, V.F. and Davidson, E.H. Evolutionary plasticity of developmental gene regulatory network architecture. *Proc. Natl. Acad. Sci. USA.* In press.

Howard-Ashby, M., Materna, S.C., Brown, C.T., Tu, Q., Oliveri, P., Cameron, R.A. and Davidson, E.H. (2006) High regulatory gene use in sea urchin embryogenesis: Implications for bilaterian development and evolution. *Dev. Biol*. **300**:27-34.

Howard-Ashby, M., Materna, S.C., Brown, C.T., Chen, L., Cameron, A. and Davidson, E.H. (2006) Identification and characterization of homeobox transcription factor genes in *S. purpuratus*, and their expression in embryonic development. *Dev. Biol*. **300**:74-89.

Howard-Ashby, M., Brown, C.T., Materna, S.C., Chen., L. and Davidson, E.H. (2006) Gene families encoding transcription factors expressed in early development of *Strongylocentrotus purpuratus. Dev. Biol.* **300**:90-107.

Istrail, S., Ben-Tabou De-Leon. S. and Davidson, E.H. (2007) The regulatory genome and the computer. *Dev. Biol*. **310**:187-195.

Juliano, C.E., Voronina, E., Stack, C., Aldrich, M., Cameron R.A. and Wessel, G.M. (2006) Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. *Dev. Biol.* **300**:406-415.

Lee, P.Y., Nam, J. and Davidson, E.H. (2007) Exclusive developmental functions of *gatae cis*-regulatory modules in the *Strongylocentrotus purpuratus* embryo. *Dev. Biol*. **307**:434-445.

Livi, C.B. and Davidson, E.H. (2007) Regulation of *spblimp1/krox1a*, an alternatively transcribed isoform expressed in midgut and hindgut of the sea urchin gastrula. *Gene Exp. Patterns* **7**:1-7.

Livingston, B.T., Killian, C.E., Wilt, F., Cameron, A., Landrum, M.J., Ermolaeva, O., Sapojnikov, V., Maglott, D.R., Buchaneen, A.M. and Ettensohn, C.A. (2006) A genome-wide analysis of biomineralization-related proteins in the sea urchin *Strongylocentrotus purpuratus. Dev. Biol.* **300**:335-348.

Materna, S.C. and Davidson, E.H. (2007) Logic of gene regulatory networks. *Curr. Opin. Biotech*. **18**:1-4.

Materna, S.C., Howard-Ashby, M., Gray, R.F. and Davidson, E.H. (2006) The C_2H_2 zinc finger genes of *Strongylocentrotus purpuratus* and their expression in embryonic development. *Dev. Biol*. **300**:108-120.

Materna, S., Berney, K. and Cameron, R.A. (2006) The *S. purpuratus* genome: A comparative perspective. *Dev. Biol*. **300**:485-495.

Nam, J., Su, Y.-H., Lee, P.Y., Robertson, A.J., Coffman, J.A. and Davidson, E.H. (2007) *cis*-Regulatory control of the *nodal* gene, initiator of the sea urchin oral ectoderm gene network. *Dev. Biol*. **306**:860-869.

Oliveri, P. and Davidson, E.H. (2007) Built to run, not fail. *Science* **315**:1510-1511.

Oliveri, P., Walton, K.D., Davidson, E.H. and McClay, D.R. (2006) Repression of mesodermal fate by *foxa*, a key endoderm regulator of the sea urchin embryo. *Development* **133**:4173-4181.

Ransick, A. and Davidson, E.H. (2006) *cis*-Regulatory processing of Notch signaling input to the sea urchin *glial cells missing* gene during mesoderm specification. *Dev. Biol*. **297**:587-602.

Revilla-i-Domingo, R., Oliveri, P. and Davidson, E.H. (2007) A missing link in the sea urchin embryo gene regulatory network: *hesC* and the double-negative specification of micromeres. *Proc. Natl. Acad. Sci. USA* **104**:12383-12388.

Samanta, M.P., Tongprasit, W., Istrail, S., Cameron, R.A., Tu, Q., Davidson, E.H. and Stolc, V. (2006) The transcriptome of the sea urchin embryo. *Science* **314**:960-962.

Sea Urchin Genome Sequencing Consortium (2006) The genome of the sea urchin *Strongylocentrotus purpuratus. Science* **314**:941-952.

Smith, J., Theodoris, C. and Davidson, E.H. (2007) A gene regulatory network subcircuit drives a dynamic pattern of gene expression. *Science* **2**:794-797.

Tu, Q., Brown, C.T., Davidson, E.H. and Oliveri, P. (2006) Sea urchin *forkhead* gene family: Phylogeny and embryonic expression. *Dev. Biol*. **300**:49-62.

Vokes, S.A., Ji, H.K., McCuine, S., Tenzen, T., Giles, S., Zhong, S., Longabaugh, W.J.R., Davidson, E.H., Wong, W.H. and McMahon, A.P. (2007) Genomic characterization of Gli-activator targets in sonic hedgehog-mediated neural patterning. *Development* **134**:1977-1989.

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Summary: Complex and intellectually challenging problems can be so commonplace that they escape our attention. The research in my lab focuses on one such everyday phenomenon - the motion of a fly through the air. While the buzz of fly wings is more likely to elicit a sense of annoyance than wonder, insect flight behavior links a series of fundamental processes within both the physical and biological sciences: neuronal signaling within brains, the dynamics of unsteady fluid flow, the structural mechanics of composite materials, and the behavior of complex nonlinear systems. The aim research in my lab is to elucidate the means by which flies accomplish their aerodynamic feats. A rigorous mechanistic description of flight requires an integration of biology, engineering, fluid mechanics, and control theory. The long-term goal, however, is not simply to understand the material basis of insect flight, but to develop its study into a model that can provide insight to the behavior and robustness of complex systems in general. The following projects in my lab, some well underway, others nascent and moving in the direction of a deeper understanding of the genetic control and brain of this insect, are helping move us in the direction of reverse engineering a fly.

29. Recording from control neurons in intact behaving animals

Alan Wong[*]

In a project in collaboration with Caltech biology professor David Anderson, post-doctoral fellow Allan M. Wong is attempting to answer a basic question of how animals are able to detect changes in their environment, decide what to do about those changes, and then coordinate their muscles to accomplish a behavioral response. We are trying to understand how these different steps are encoded in the nervous system of the fly. While these are large questions, we think a good entry point is to understand the function and connectivity of descending interneurons - the neurons that connect the brain with a fly's thoracic ganglia and thus are the equivalent of the spinal cord in vertebrates. In the past year, senior post-doctoral fellow Will Dickson and post-doctoral fellow Andrew Straw have helped us to construct a useful arena in which to study fly behavior: a tethered walking ball, first developed by Erich Buchner 30 years ago in Germany. In this device, a tethered fly walks on top of a floating styrofoam ball. The experimental setup simulates walking behavior while keeping the fly stationary, which allows us to conduct tightly controlled stimulus-response experiments that can be coupled with functional imaging via 2-photon microscopy and electrophysiological recordings to monitor neural activity. We have also developed a surgical preparation where the cuticle is removed from the fly head to permit two-photon imaging in the fly. Flies with these surgical procedures can still respond to visual stimuli and walk normally within the arena.

[*]*Postdoctoral Scholar, Anderson lab, Division of Biology, Caltech*

30. The role of visual and mechanosensory cues in structuring forward flight in *Drosophila*

Seth Budick

Another area of work in my lab is the development of autonomous miniaturized flying robots. Our hope is to better understand the rules that govern insect flight and then apply some of these rules, where appropriate, to the engineering of micro-flying robots. Much of this work has involved studying the role of visual cues in insect flight. The involvement of mechanosensory input -- the way flies detect their own speed relative to the surrounding air and some directional deviations - has been little studied. Graduate student, Seth Budick, who was awarded his Ph.D. this summer, tested the role of mechanosensory cues in fly flight. He did this by tethering flies to steel pins that were suspended between two magnets. This preparation allowed the flies to rotate freely about one axis (**Figure 1**). We found that flies orient into oncoming winds, as would be experienced during flight, and they orient more strongly as wind velocity increases. Additional experiments revealed that the response is mediated by the Johnston's organs, a set of paired organs located in the antennae and known to be involved in sound detecting. Removal of both of these organs resulted in a complete loss of orientation ability while restoration of a single organ partially rescued the orientation response. The work also explains the paradox why free-flying flies do not avoid expanding visual flow fields (which look like large objects looming toward them) while tethered flies do. Mechanosensory cues are capable of suppressing the expansion.

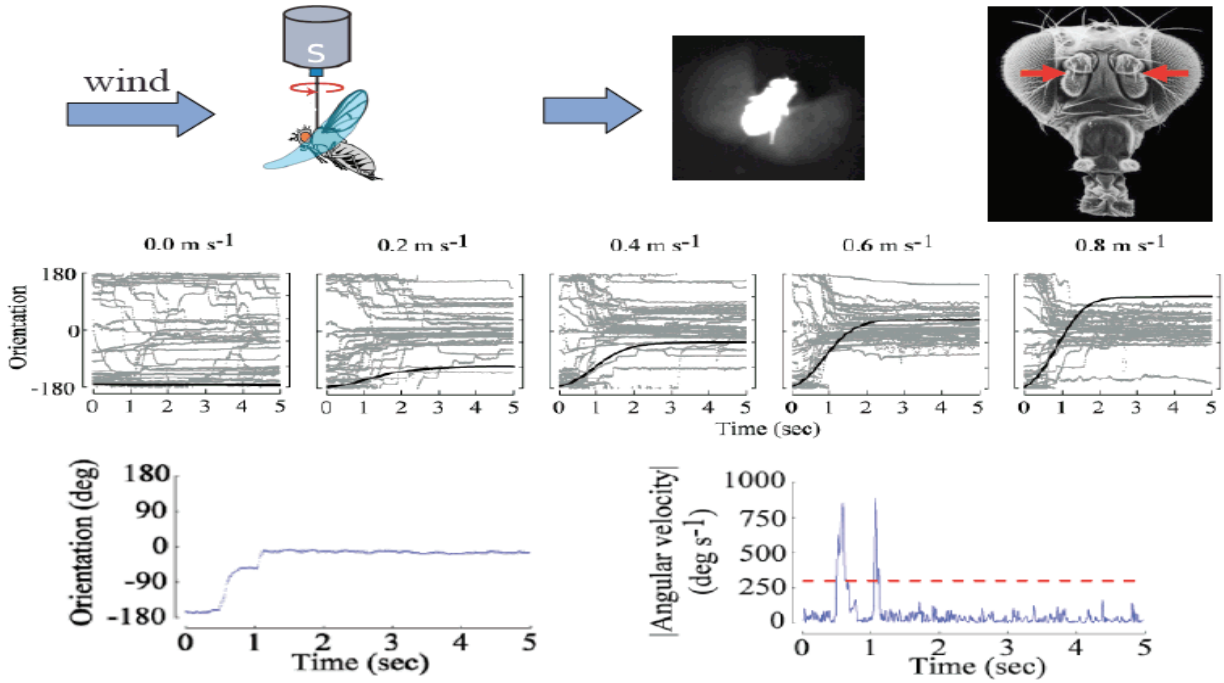

Figure 1. Summary of flight responses to combination of mechanosensory and visual cues. The fly is tethered to a steel pin that is free to rotate within a magnetic field. Unidirection flow is generated by a wind tunnel. A automated camera system captures the fly's orientation. The second row of data show the flies' orientation n response to wind of increasing strength. Whereas the initial orientation is random, the flies orient into the wind once the flow is turned on. The bottom row of figures indicates that the flies often orient by making quick saccadic turns.

31. The role of passive mechanisms in controlling wing rotation in insect flight

Will Dickson

Our lab is also continuing to study the aerodynamics of insect flight in order to better understand how these animals accomplish their impressive aerial locomotion and also to aid in the development of miniaturized flying robots. Studies led by senior post-doctoral fellow, Will Dickson, have focused on wing rotation. Rapid rotation of the wings allows for lift generation during both upstroke and downstroke during flight. Depending on when it occurs during the stroke cycle, wing rotation can either increase or decrease the aerodynamic forces produced by the wings. One thing that is not well understood is the degree to which insects employ passive vs. active mechanisms to rotate their wings during flight. In order to help understand the primary factors influencing the timing and magnitude of passive wing rotation, we developed a dynamic model of a flapping wing that includes both inertial effects and aerodynamic forces. This model enables us to examine the effects of mass distribution, wing shape, rotational axis location and driving function. We are also using two mechanical models to examine the physics of passive wing rotation and to provide experimental validation of the dynamic model (**Figure 2**). These include a large robotic wing flapping in a tank filled with mineral oil (to simulate a smaller wing flapping through air) and a small 3-inch model wing flapping in air at high frequencies. The basic find is that passive mechanisms are capable of generating a time course of wing rotation that is quite similar to that generated by a real fly.

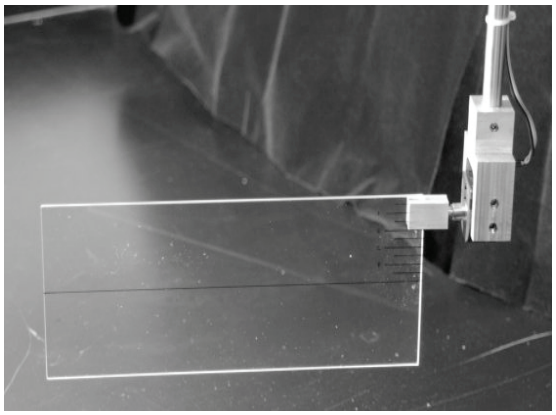

Figure 2. Robotic test wing for studying the role of passive mechanisms in generating the changes in angle of attack during the wingstroke.

32. The active control of haltere motion in flies

John Bender

One feature distinguishing the true (dipteran) flies from other insects is the mechanosensory organs called halteres, small club-shaped appendages evolved from the hindwings. Like gyroscopes, the halteres are sensitive to Coriolis forces generated during a fly's body rotations. Most investigations of haltere function have focused on their well-established role as negative feed back connections to maintain flight equilibrium, but as serial homologs of the forewings, the haltere system also includes muscles that could allow the animal to exert active control of this feedback. However, such alteration of the haltere dynamics during flight has never been demonstrated. We investigated this question by using an optical wing beat sensor to detect the wing strokes in real time and monitor the wing beat period on a stroke-to-stroke basis. This signal was used by a computer to predict the wing beat period for each upcoming wing stroke, from the average of a few previous strokes and trigger a strobe and camera to collect phase-locked images of the halteres at precise points in the wing beat cycle. These videos, phase-locked to the wing strokes, show that although the halteres and wings beat synchronously in antiphase, the stroke position of the halteres relative to the wings is not constant from stroke to stroke. To manipulate the fly's flight, we provided visual stimulation using patterns simulating pure rotation about each of the yaw, pitch, and roll axes. Flies respond to such visual stimuli by asymmetrically altering their wing strokes in patterns that would rotate the fly syndirectionally with the visual motion. Generally, this corresponds to increasing the wing beat amplitude of one wing while slightly decreasing the amplitude of the contralateral wing stroke. Our video analysis showed that haltere stroke amplitude is strongly correlated with the contralateral wing stroke amplitude. This demonstrates that the haltere strokes are actively modulated during flight, and that this modulation is dependent on the fly's attempts to turn. This suggests that haltere feedback is activated by descending control signals, possibly either as an efference copy used to cancel reafferent feedback from the halteres during active turns, or to initiate turns by actively generating haltere sensory signals which lead to robust flight responses.

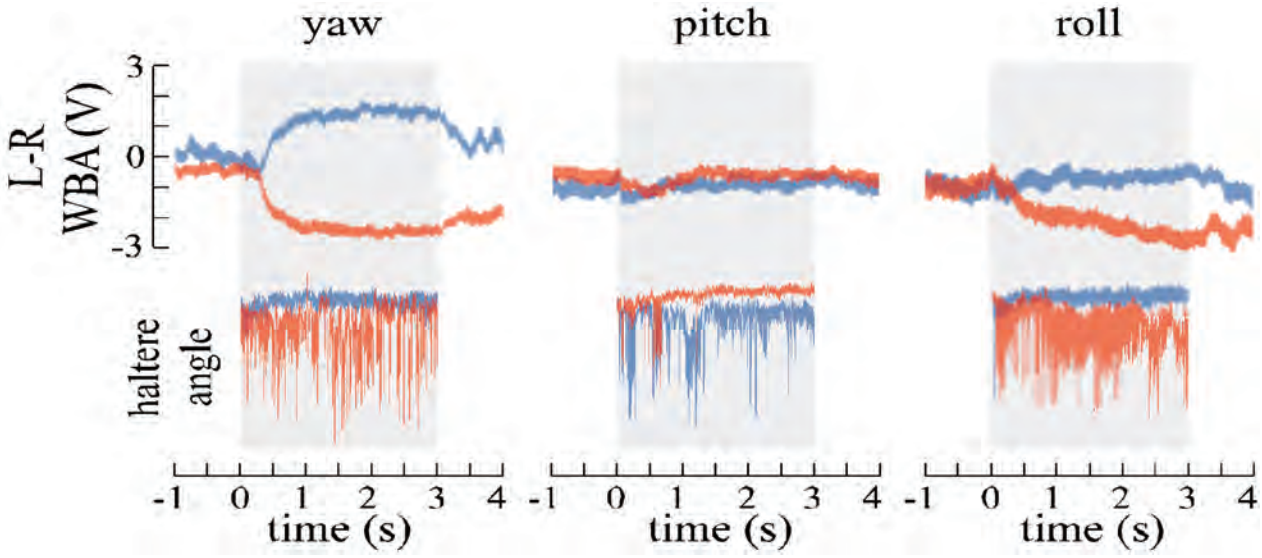

Figure 3. Haltere motion is altered during compensatory responses. The top traces show the changes in wing motion induced by visual rotations about the yaw, pitch, and roll axes. The bottom traces (haltere angle) show changes in haltere stroke angle.

33. Development of insect-inspired flight control algorithm

Sawyer Fuller[*]

The lab is also using control systems theory to generate models of fly flight that incorporate the latest findings from work using free-flight tracking, computational aerodynamics, genetics and neuroscience. Such models need mathematical and physical environments for testing. One such platform is the Grand Unified Fly model (GUF), which incorporates a 3D visual environment, a visual system simulation, and kinematic and aerodynamic models of flapping flight, written by William Dickson and Andrew Straw of the Dickinson Lab. The second is Flysight, an adaptation of the Caltech Multi-Vehicle wireless testbed that incorporates a fan-actuated hovercraft robot with a sensory system that emulates the fruit fly. Undergraduate Sawyer Fuller, a student in the CDS option, is working on this analysis. On Flysight we implemented a corridor-following algorithm that uses optic flow estimated by Hassenstein-Reichardt Elementary Motion Detectors (EMD's) on infrared sensors imaging the wall (see **Figure 4**). The algorithm is a refinement of J. Sean Humbert's (Ph.D., Caltech, 2005) that adapts it to EMD's. The robot navigates the corridor, though not robustly. In GUF we implemented a longitudinal flight controller that stabilizes forward, vertical, and pitch motion. Controller inputs are velocity estimates are from EMD's and halteres and outputs are wing stroke amplitude

(thrust) and mean stroke position (pitch torque). The controller design stabilized a linearized model of the stroke-averaged aerodynamic behavior. We also developed a geometric analysis of EMD's to enable the use of analytic tools of control theory. The approaches so far have resembled traditional controllers, but there is no reason to believe flies encode such concepts as velocity, so one goal is to identify the fly's controller.

We are building an apparatus to combine free-flight tracking with wind gusts to observe the fly's disturbance rejection dynamics to progress in this direction.

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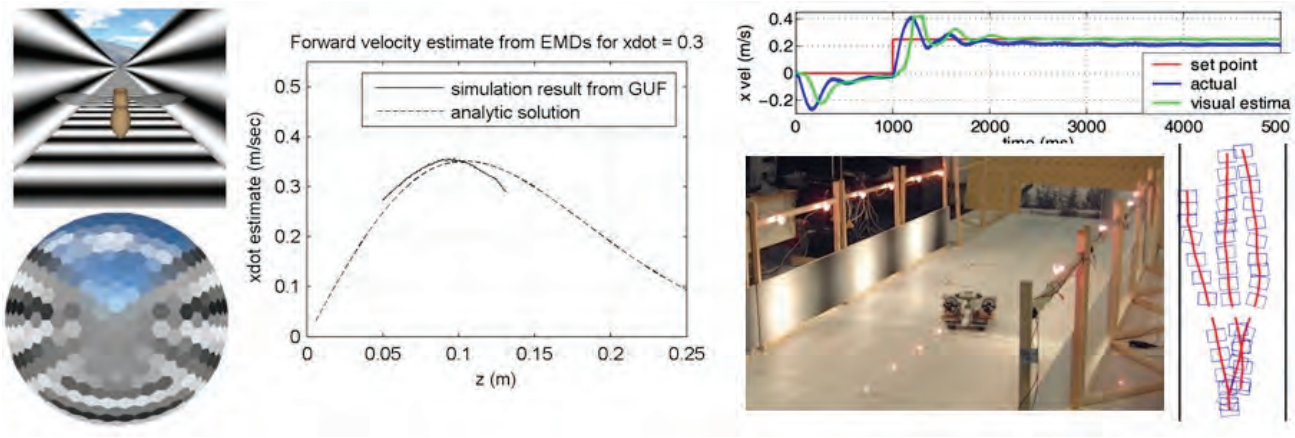

Figure 4. Mathematical (GUF) and physical (FlySight) testbeds for studying visual control algorithms of insects. The left panels show examples of the GUF environment (from the fly's point of view and from an external reference). A forward velocity estimate is derived from an array of elementary movement detectors. The panels on the right show FlySight, with a hovercraft navigating down a corridor using a fly-inspired visual control algorithm.

34. The predator vs. perch visual detection system of *Drosophila*

Gaby Maimon

One very exciting new line of research in the lab has been conducted with post-doctoral researcher Gaby Maimon. Gaby has discovered what appears to be a simple visual algorithm that underlies attraction and repulsion decisions in fruit flies. A wide-range of insects adjust their locomotion so as to approach long vertical edges, a behavior called 'fixation.' We examined how this orientation response changes, as the stimulus is made progressively shorter. The data indicate that very small visual stimuli, spanning only a few rows of ommatidia in the fruit fly eye, elicit not weak fixation, but strong repulsion. Specifically, in tethered-flight we observed that flies turned toward the mean location of a long vertical stripe, but consistently turned away from a shorter stimulus (**Figure 5**). In closed-loop experiments – in which tethered flies themselves controlled the movement of the stripe – we found that flies kept long stripes in front of their line of flight (fixation), but they tended to keep short stripes behind their line of flight (anti-fixation) or allowed short stripes to spin unstably around the arena (spinning). Consistent with the tethered-flight data, 3D tracking of freely flying *Drosophila* showed that they approached long vertical posts, but the animals tended to steer clear of small objects in their immediate vicinity. The differential response to long vertical edges and tiny spots may reflect a simple but effective object-recognition system. Fixation would attract insects toward vegetative perches whereas repulsion from tiny stimuli would help them avoid aerial predators or impending collisions. *Drosophila* flight behavior is likely to depend on a balance of these two systems – one attractive, the other repulsive – providing a foundation for studying the neural basis of object identification and behavioral choice in a genetic model organism.

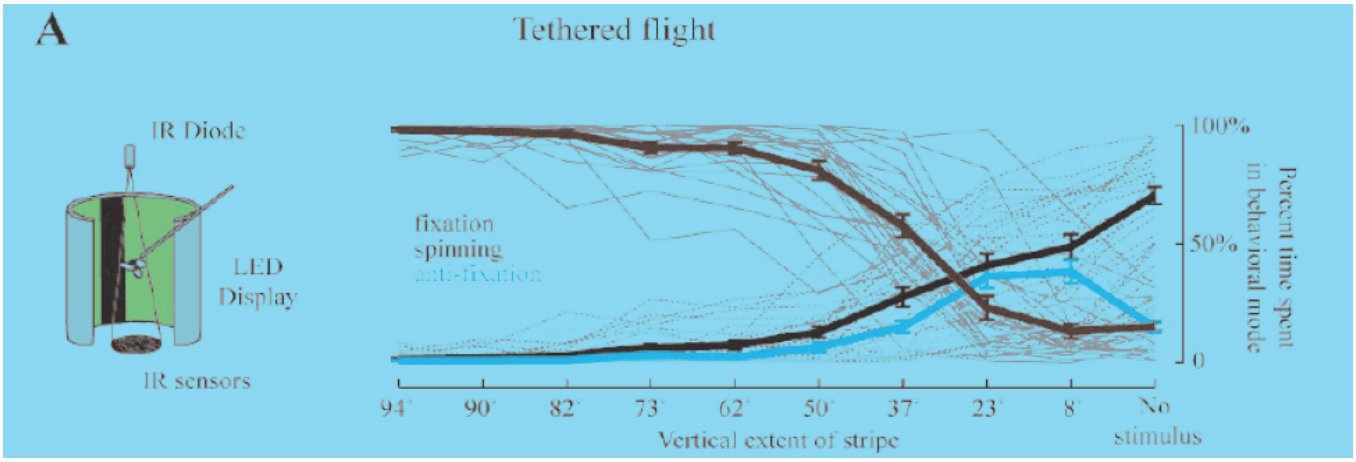

Figure 5. Closed-loop fixation responses of tethered flies as a function of stripe length. Flies actively fixate (head towards) long stripes, but they either anti-fixate (steer away from) small stripes.

35. Decision-making algorithms in flying *Drosophila*

Andrew Straw

Work on the questions of decision-making in flies is also being conducted by postdoctoral researcher Andrew Straw, who is trying to determine how flies decide whether or not to land on an object. Many insects fixate and approach vertical features in their visual environment while walking or flying. We have investigated this behavior in freely flying *Drosophila* within an arena containing a single vertical post. Flight trajectories indicate that flies are strongly attracted toward this feature compared to flights in which the post was removed. Once the flies get very close to the post, however, they turn and fly away (**Figure 6**). If the source of an attractive odor (apple cider vinegar) is present on the post, the tendency to turn is strongly decreased and flies land on the probability of landing on the post is greatly increased. Thus, attractive odor influences or overrides the visual control of turning in the immediate vicinity of a vertical post. As a first step in exploring the mechanistic underpinning of this behavior, we are developing algorithm-level, phenomenological models of flight behavior based on simple models of visual processing and flight dynamics. Critical parameters of the models are derived using system identification techniques in conjunction with the extensive data sets collected using a custom-built automated tracking system. Although such simple control models are relatively easy to construct and analyze, they can provide only limited insight into the underlying neural circuits. Therefore, as a further step towards understanding the neural basis of these behaviors, we are also employing a detailed model of *Drosophila* visual physiology coupled with physically realistic models of flight aerodynamics. Using this bottom-up simulation environment, we are attempting to create biologically plausible controllers that give rise to similar behaviors. Such bottom-up models will be of practical use in conjunction with electrophysiology, neural activity imaging, and molecular genetic manipulations to identify and characterize the neural circuits underlying object fixation, approach, and odor-modulated decision making.

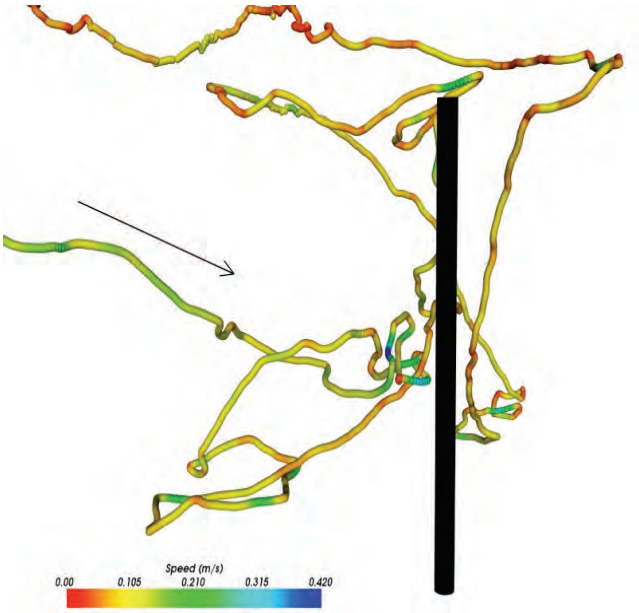

Figure 6. Flight trajectory of flying fruit fly as it approaches, investigates, and flies away from a post laden with vinegar odor. The flight velocity is encoded by pseudocolor. Data were collected using the Flydra system of real-time 3D tracking.

36. Decision-making in hungry flies

Jasper Simon

Another goal of the lab is to find a way to automate laboratory studies of behavioral phenotypes in fruit flies so that this powerful genetic model organism would also be a powerful tool for studying more complicated behaviors. To aid in this we have developed a flexible system of experimental chambers that we have dubbed "Flyworld." Graduate student Jasper Simon is now using Flyworld to determine why fruit flies disperse away from food sources. We have previously reported that a

requirement for food and not cues from food determine dispersal. Moreover, we observed that mating stimulates both male and female *Drosophila* to disperse from chambers without food at a higher rate than virgins, and that mating inhibits female *Drosophila* to disperse from chambers that contain food. While the presence of food inhibits the dispersal of hungry male and female *Drosophila*, we have recently observed that groups of hungry male *Drosophila* disperse from chambers without food at a higher rate then groups of hungry female *Drosophila*. Could the results from our dispersal experiments be explained by a change in the level of general activity? To test this hypothesis we used the commercially available *Drosophila* activity monitor (TriKinetics Inc). Using these activity monitors we observed a difference in the activity between male and female *Drosophila*, consistent with the results from the dispersal experiments carried out in our experimental chambers (**Figure 7**). Interestingly, when we attempted to test the effects of hunger and mating history on activity we repeatedly observed spurious results. These unpredictable results give reason for concern when evaluating studies carried out with this commonly used technology. As an alternative to experiments carried out with the *Drosophila* activity monitors we have developed a computer-camera system to track individual *Drosophila* within our experimental chambers.

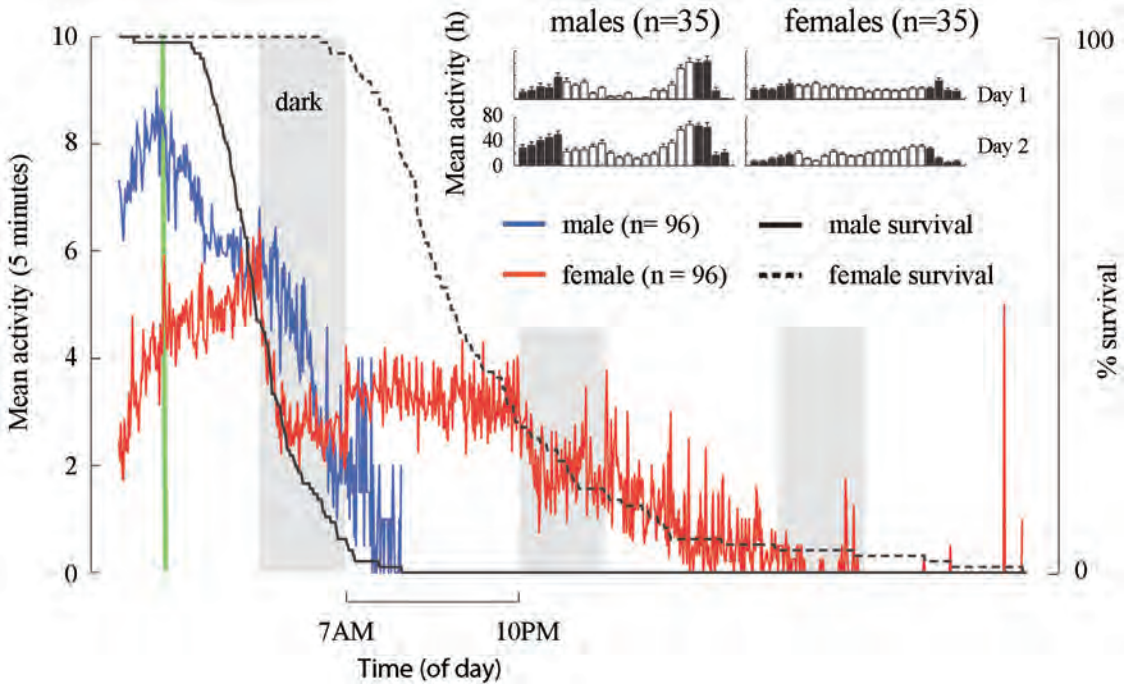

Figure 7. Here we show means from the general locomotor activity and survival of individual male and female *Drosophila*. Individuals had access to water throughout the experiment. At three hours into the experiment (green, vertical line), males (blue; 8.25+/-0.29 SEM) exhibited a significantly higher level of activity then females (red; 4.14+/-0.21 SEM), t-test; $p<0.0001$. Gray bars denote entrained dark light cycle. Inset: We report the average, hourly crepuscular activity from male and female *Drosophila* monitored for two days.

37. VialCam: An automated behavioral analysis chamber for fly behavior

Wyatt Korff

Another relatively simple system we have developed to perform an inexpensive high throughput behavioral analysis system for fruit flies is *VialCam*. The system can track and quantify the motion of flies as they behave and interact within their standard laboratory habitat – a commercially available food vial. The Dickinson Lab has successfully created working hardware prototypes for VialCam out of ABS plastic on our CNC milling machine and have begun software development for VialCam. The hardware consists of an inexpensive USB-bus powered web camera with three white LEDs that control photoperiod independently of the three near infrared LEDs used for illumination during video acquisition. A wide-angle lens with a 185-degree field of view directly couples the video camera to VialCam. VialCam securely slips over the end of a standard *Drosophila* vial replacing the stopper. Two airports are available to pump in odors for olfactory experiments or oxygen sensors for physiology studies. We have written acquisition software in the MATLAB programming environment to stream video sequences to disk for offline processing. The bottom and sides of the vial are visible and in relatively clear focus. Because *Drosophila* cuticle reflects nIR light, when illuminating with a nIR light source, animals show up as bright white objects against the background. After applying a simple background subtraction algorithm to compensate for non-uniform illumination and a modified

particle-tracking algorithm, we have successfully resolved and tracked the center of mass of up to twenty individual flies at one time. Unfortunately, flies 'shadows' also reflect off of the interior of the vials, leading to inaccurate center of mass estimates. We are working on improving the image processing software as well as experimenting with anti-reflective coatings for the inside of the vials to improve tracking. With appropriate image processing software, this simple system will provide basic quantitative information relating to many behaviors such as locomotion, grooming, feeding, and the simpler components of courtship and aggression.

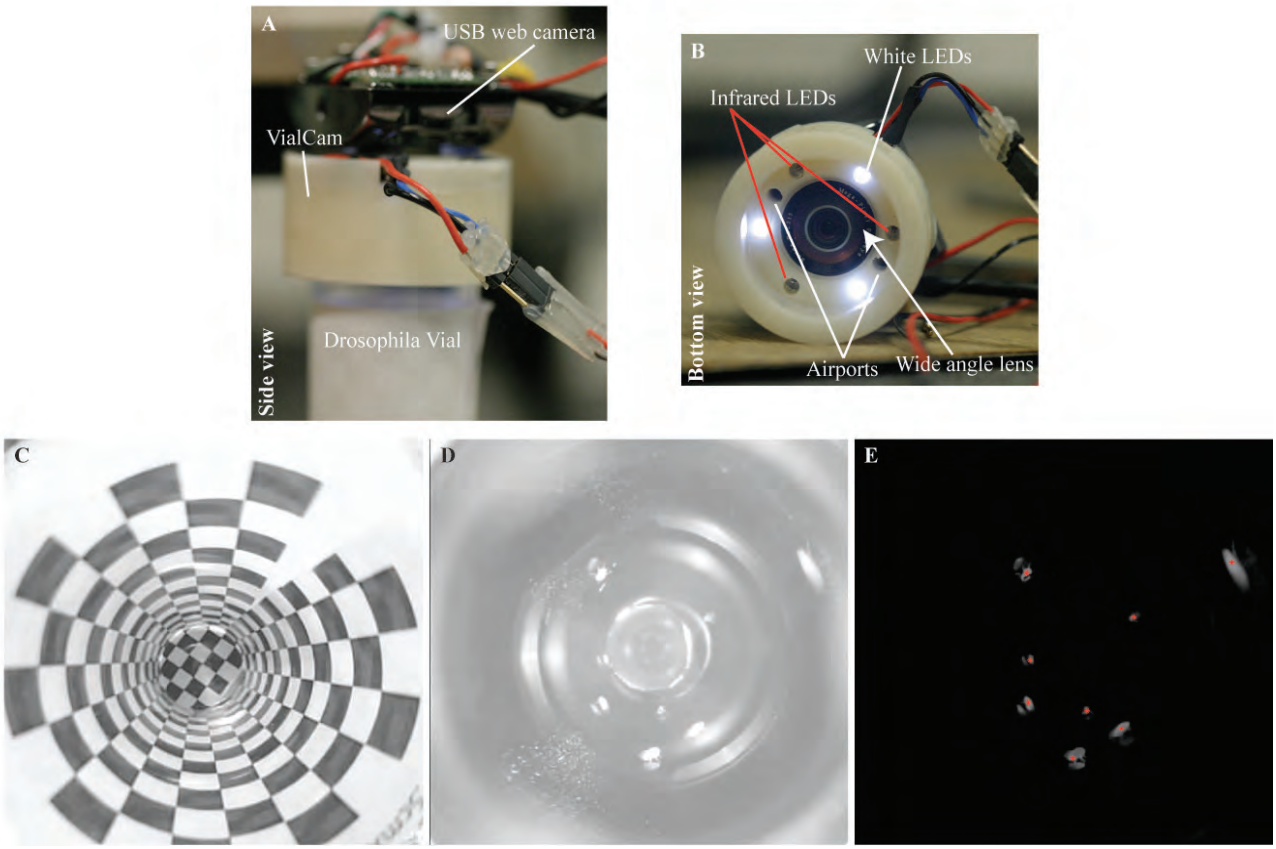

Figure 9. VialCam prototype. Two images outside (A,B) and three images inside (C,D,E) of VialCam. The entire module will fit inside of standard *Drosophila* vials to provide a cheap and high-throughput analysis of fly behavior.

Publications

Bender J.A. and Dickinson M.H. (2006) A comparison of visual and haltere-mediated feedback in the control of body saccades in *Drosophila melanogaster*. *J. Exp. Biol.* **209**:4597-4606.

Budick, S.A., Reiser, M.B. and Dickinson, M.H. (2007) The role of visual and mechanosensory cues in structuring forward flight in *Drosophila melanogaster*. *J. Exp. Biol.* In press.

Dickson, W.B., Straw, A.D., Poelma, C. and Dickinson, M.H. (2006) An integrative model of insect flight control. *AIAA Journal*. In press.

Reiser, M.B. and Dickinson, M.H. (2007) A modular display system for insect behavioral neuroscience. *J. Neurosci. Meth.* In press.

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Summary: Cells process information, interact with one another, and exhibit patterned development using circuits composed of interacting genes and proteins. Although many of these components and their interactions are now known, it remains unclear how the circuits they compose function reliably within cells. In order to understand how genetic circuits operate at the single-cell level, we are applying experimental and theoretical techniques to key model systems:

First, we construct synthetic genetic circuits and study their behavior in individual cells. These synthetic circuits are simpler counterparts to the complex circuits one finds in nature. This approach, often called "synthetic biology," allows one to analyze how various circuit designs might work, and begin to understand what is special about the specific circuit architectures observed in organisms. We have constructed circuits that exhibit oscillations and other dynamic phenomena, (e.g., Elowitz & Leibler, 2000). We have also used synthetic circuits to analyze the dynamics and variability of gene regulation at the single-cell level, (e.g., Elowitz *et al*., 2002, and Rosenfeld *et al*., 2005). We also make use of 're-wiring' perturbations to alter the architecture of natural genetic circuits. Current synthetic biology projects focus on regulation of two-component systems in bacteria, and higher-level developmental pattern formation in mammalian cell culture.

Second, we analyze the dynamics of specific natural genetic circuits in order to understand basic principles of their operation. We have developed the ability to acquire and quantitatively analyze large time-lapse movie datasets. These movies allow tracking of circuit dynamics in individual cells as they grow and develop. By incorporating several distinguishable fluorescent protein reporter genes in these organisms, we can track multiple circuit components simultaneously. The results constrain models of the corresponding circuits and provide insight into basic principles of their operation. A recent example of this approach is our work on regulation of genetic competence in *Bacillus subtilis* (see Süel *et al*., 2006 and Süel *et al*., 2007).

Third, we are analyzing the generation of variability within cell populations. Genetically identical cells appear to actively generate variability, even in homogeneous environmental conditions. We focus specifically on two complementary questions: How do cells use intrinsic "noise" (stochasticity) in their own components to make effectively random cell fate decisions? And how do they suppress noise in order to operate reliably despite of variability. Current projects are examining these issues in *Bacillus subtilis*, a very simple prokaryote that exhibits both differentiation and development, as well as in more complicated mammalian cell culture systems.

Projects in the lab make extensive use of relatively simple mathematical models of genetic circuits. We are also developing software and tools to improve gene circuit construction and quantitative analysis of movie data.

38. Noise in signaling of single cells

Long Cai, Michael Elowitz

In eukaryotic cells gene regulation is accomplished partly through regulation of nuclear localization of transcription factors in response to extracellular signals. Although the molecular mechanisms of nuclear import and export have been elucidated, it remains unclear how localization varies among individual cells, and how dynamic changes in localization affect expression of downstream genes. In the presence of extracellular calcium, Crz1p, the calcineurin responsive zinc finger transcription factor of *Saccharomyces cerevisiae*, is dephosphorylated and translocated into the nucleus. The signaling dynamics of this system can be observed in individual cells by tracking the localization of Crz1-GFP fusion proteins in time-lapse fluorescence microscopy. We observe that, in the presence of external calcium, Crz1p localizes to the nucleus in coherent bursts which a characteristic nuclear residence time of ~1 minute. These localization bursts occur stochastically in time and increase in frequency as concentration of external calcium increases, without a change in mean residence time. Localization dynamics vary widely among cells, as measured by autocorrelation functions determined in individual cells. Msn2p, a general stress response transcription factor that exhibits similar localization burst behavior in the presence of calcium. However, the localization bursts of Crz1p and Msn2p are uncorrelated, suggesting that nuclear localization bursting behavior is

not driven by a common "upstream" source of fluctuations. Finally, we show there is a correlation between Crz1p localization events and the expression of downstream Crz1p-responsive genes in single cells, indicating that stochastic localization bursts contribute significantly to fluctuations in gene expression in stress response genes.

39. Noise-dependent developmental plasticity and the evolution of novel morphologies in *B. subtilis*

Avigdor Eldar[*], Vasant K. Chary[1], Panagiotis Xenopoulos[1], Jonathan Dworkin[2], Patrick Piggot[1], Michael Elowitz*

Developmental processes can change, sometimes drastically, in response to environmental conditions or mutations. A central question in evolution is how this developmental 'plasticity' is related to the evolution of new morphologies over longer timescales. *B. subtilis* sporulation represents an ideal model developmental system to address this problem. In *B. subtilis*, sporulating cells each produce a single mature spore through a process that involves asymmetric division into forespore and mother cell compartments. Here we use time-lapse fluorescence microscopy on individual cells to quantitatively analyze how sporulation depends on inter-compartmental signaling and other events. We find that when signaling is delayed, cells employ a spectrum of "backup" responses, which are activated in different sub-populations. These responses include a previously unknown process in which chromosome replication re-initiates, leading either to an additional sporulation attempt or to vegetative division. When signaling is sufficiently delayed, a fraction of the population exhibits the simultaneous development of two "twin" spores from a single cell. Twin sporulation in *B. subtilis* appears similar to multi-spore development in other, "polysporogeneous," microbial species. By analyzing the timing and amplitude of gene expression, inter-compartmental signaling, and chromosome replication and translocation events in individual cells of both wild-type and mutant *B. subtilis* backgrounds, we suggest molecular mechanisms responsible for twin spore development, and predict specific additional mutations that increase its penetrance. These results show, at the molecular level, how regulatory perturbation of a single gene can reveal underlying developmental plasticity and how further mutations can stabilize this alternative developmental program, allowing the evolution of a new morphological program.

[*]*Equal contribution*

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40. Global regulatory dynamics of *B. subtilis*

James C. Locke, Michael Elowitz

Clonal populations of cells contain the same genome, and may be grown in the same environment. However individual cells are frequently found to exhibit a heterogeneous set of cellular states. A fundamental question in biology is how this heterogeneity is generated. Recent work suggests that stochasticity, or noise, in underlying reactions is used by cells for probabilistic differentiation. Thus, a major problem is to understand, first, how such noise enables heterogeneous decision-making in cell populations and, second, how genetic circuits within the cell constrain variability to generate specific cellular states.

We are using *B. subtilis* as a model system to study this problem. This bacterium exhibits a broad range of differentiated states even in a single environment, including terminal and transient differentiation states (sporulation and genetic competence, respectively) as well as an array of metabolic and regulatory states that are activated in a subset of cells and may persist for more than a cell generation. These states are often controlled by master transcription factors, facilitating analysis with fluorescent protein reporter genes.

We are combining experiments at the single-cell level and theoretical modeling to identify the set of possible states in *B. subtilis*, and investigate their interactions. We have constructed a set of strains with multiple fluorescent reporter genes and are currently analyzing the modularity and compatibility of specific differentiated states, and the dynamic transitions among them.

41. miRNA regulatory dynamics at the single-cell level

Georg F. Seelig, Michael Elowitz

Micro RNAs regulate on the order of 30% of all protein-coding genes in humans and provide an important mechanism for post-transcriptional gene regulation. The protein machinery involved in the processing of miRNA is by now fairly well characterized. In contrast, relatively little is known about cellular functions of specific miRNAs and about the dynamics of gene regulation by miRNA. These two questions seem related, as a better grasp of *in vivo* regulatory dynamics should further our understanding of their potential biological roles.

I am currently investigating miRNA regulatory dynamics on the single-cell level using fluorescence time-lapse microscopy and using an mRNA coding for a fluorescent protein as a synthetic target for the regulatory RNA.

The questions I hope to answer are: (i) what are the time-scales for repression and derepression of a target gene product by a miRNA and how much do repression and derepression dynamics vary from cell to cell? (ii) MicroRNA can regulate their targets through a variety of mechanisms. Can we distinguish different mechanisms of gene regulation by miRNA and can we extract *in vivo* biochemical parameters for a given mechanism?

42. Dynamic of Notch signaling in single cells

David Sprinzak, Graham Anderson, Michelle Fontes, Michael Elowitz

Notch signaling functions in developmental patterning processes, such as lateral inhibition patterning and boundary formation. Although many of the molecular details of the Notch signaling pathway have been elucidated, systems-level parameters critical for the proper function of Notch-based developmental circuits remain unknown. For example, relevant time scales and time delays in the signaling process are critical for the proper function of lateral inhibition circuit. Here, we use fluorescent time lapse microscopy on mammalian cells to follow the dynamics of Notch signaling at the single-cell level. To quantitatively measure Notch signaling dynamics, we use modified Notch1 receptors that have Gal4-VP16 transactivator domains. We simultaneously monitor the downstream transcriptional response of Notch1 targets using fluorescent protein reporters. We show that we can follow quantitatively over the dynamics of the Notch signaling. We measure the time delay between onset of signaling and the start of a transcriptional response. We discuss the possible applications of this technique and its implications in understanding and programming developmental patterning.

43. Programming promoter logic

Robert Sidney Cox III, Michael G. Surette[*]*, Michael Elowitz*

We have investigated the relationship between promoter architecture and gene expression using a combinatorial promoter library. Here, the placement, affinity, and sequence of known binding sites were systematically varied, allowing us to determine the range of functions encoded by the simplest combinatorial promoters. Promoters were assembled by ligating oligonucleotides corresponding to 16 variants of each of three promoter regions: *distal*, *proximal*, and *core*. Of the 16 variants of each promoter region, 11 contained binding sites to one of four transcription factors; the five remaining variants contained no binding sites. The four transcription factors investigated were the activators AraC and LuxR and the repressors LacI and TetR. Promoters could thus contain up to three binding sites for different transcription factors. All assembled promoters were linked to luciferase gene expression. A subset of 288 randomly chosen promoters were sequenced and their expression patterns were probed over all 16 combinations of the four inducers.

Because of the continuous nature of the output levels in each input state, Boolean logic does not accurately represent all possible promoter functions. Therefore, we introduced an intuitive three-dimensional logic parameterization for the space of promoter functions. In this scheme, we represented promoter phenotypes with three numerical parameters that quantify dynamic range, logic type, and asymmetry (Fig. 1). We define r as the ratio of the maximum to minimum expression level. Second, the parameter l quantifies the logical behavior of the gate: from pure OR (l = 0) to pure AND logic (l = 1). Third, the parameter a quantifies the asymmetry of the gate with respect to its two inputs. At a = 0, the gate responds symmetrically to either inducer, while at a = 1, the promoter responds only to a single input.

Combinatorial promoter synthesis permits systematic perturbation of promoter architecture and rapid identification of sequences that implement specific functions. The spectrum of promoter functions observed here highlights several heuristic rules for promoter design: (1) *Unlimited regulation*. Regulated promoter activity is independent of unregulated activity; (2) *Repression trend*. The effectiveness of repression depends on the site with *core* ≥ *proximal* ≥ *distal*. Following this trend, RR-promoters may be symmetric or asymmetric; (3) *One is enough*. Full repression is possible with a single operator between -60 and +20. Activators function only upstream of -35 (*distal*), and have little effect downstream (*core* or *proximal*); (4) *Repression dominates* activation, producing asymmetric AR-promoter logic and, (5) *Operator proximity.* Separation of input variables generates SLOPE and asym-SLOPE logic only. Moving operators closer together makes the logic more AND-like.

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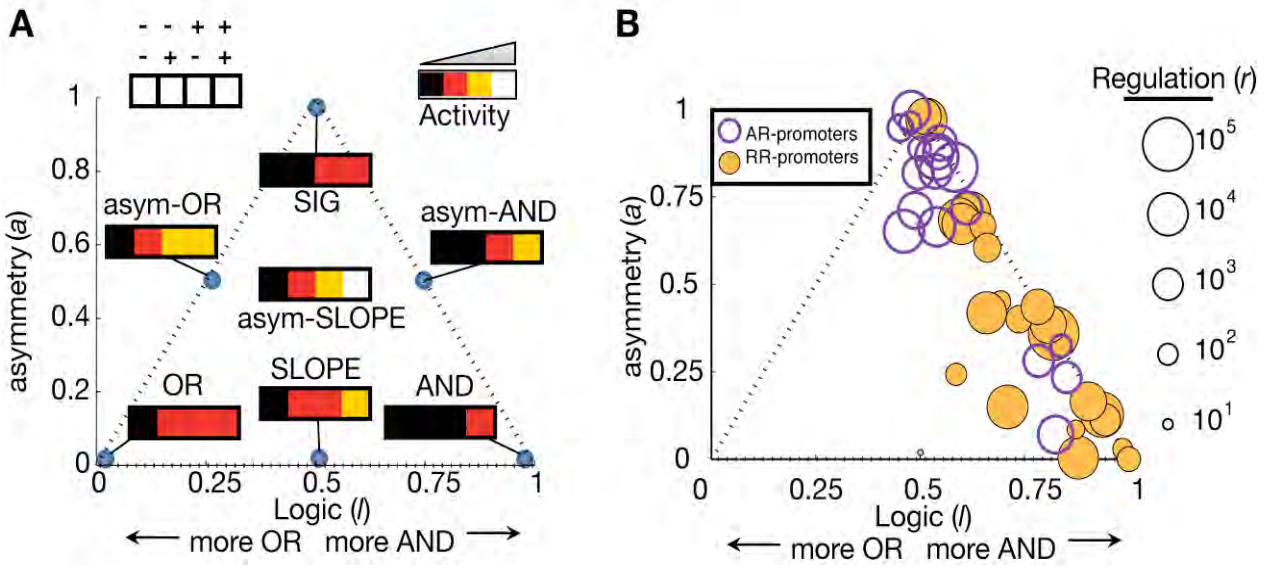

Figure 1. Dual-input gates in logic-symmetry space. (A) Diagram showing the space of allowed logical phenotypes, with the locations of Boolean and intermediate logic gates indicated. The SIG gate responds completely to one inducer and not at all to the other. The SLOPE gate represents an intermediate logical function between AND and OR, while the asymmetric gates represent intermediates between SIG and the corresponding symmetric gate. (B) The logical phenotypes of 50 dual-input promoters exhibiting strong regulation ($r \geq 10$). AR-promoters are shown as purple circles, RR-promoters are shown as gold disks. The diameter of each disk is proportional to the logarithm of its regulatory range, r.

44. The effects of evolutionary selection on noise

Chiraj K. Dalal, Michael Elowitz

Many biological traits are quantitative: levels of gene expression, sizes of appendages, and abundances of cellular components can vary over a wide range. Mean values of such phenotypes are generally genetically encoded; therefore, they are subject to the forces of selection. Recently, it has become clear that such phenotypes are fundamentally noisy: that is, genes specify a distribution of possible values for the trait, rather than a precise value. More significantly, the variance of this distribution, like the mean, is under genetic control [1], and the variance, at least in stress response genes, may be genetically independent of mean [2][3]. This data suggests that both the mean and variance of a quantitative phenotype are influenced independently by selective pressures that act on the phenotypes expressed in individual cells. However, an individual in a population is subject to selection only on its particular phenotypic value, not on the mean and variance that specify its phenotypic distribution. Here we ask how positive directional selection affects variance in a simple quantitative trait.

Using a simple population genetics model of mutation, selection and growth, we find that noise increases under strong selection and decreases under weak selection. Furthermore, we find that the increase in variance is proportional to mutation rate and that the general result applies to other measures of phenotypic diversity. However, these theoretical conclusions are based on two main assumptions: First, we assume that noise and mean expression levels vary independently with respect to mutations. Second, we assume that small mutations can add to produce larger changes in both mean and noise and that mutations of very large positive effect are rare. We are continuing to further analyze theoretically how these and other assumptions affect the evolution of noise.

Furthermore, we are in the process of experimentally verifying both these assumptions. Using gene expression as a quantitative phenotype, we created three promoter-YFP strains and measured mean and variance of gene expression using flow cytometry. We have created mutant libraries for these three promoters and have found that mean and variance appear to be independent in some cases. We are currently in the process of testing the additivity of mutations, our second assumption, by remutagenizing individual mutants in the library. Finally, we hope to pool the mutants in one library together and use flow cytometry to artificially impose phenotypic selection on the mutant- tracking the changes in mean and variance of gene expression as they evolve.

References

[1] Raser, J.M. and O'Shea, E.K. (2004) *Science* **304**:1811-1814.

[2] Bar-Even, A., Paulsson, J., Maheshri, N., Carmi, M., O'Shea, E., Piplel, Y. and Barkai, N. (2006) *Nature Genet.* **38**:636-643.

[3] Newman, J.R., Ghaemmaghami, S., Ihmels, J., Breslow, D.K., Noble, M., DeRisi, J.L. and Weissman, J.S. (2006) *Nature* **441**:840-846.

45. Mechanisms of probabilistic commitment in a prokaryotic developmental circuit

Joseph Levine, Michael Elowitz

When confronted with extreme environmental or nutritional stress, the gram-positive bacterium *Bacillus* subtilis initiates a developmental program causing it to develop into a environmentally resistant spore. We have observed that this developmental process shows considerable variability in both the timing and patterns of gene expression. Using single-cell time-lapse microscopy and an array of fluorescent reporters in various background strains, we are working to understand the gene expression mechanisms underlying this behavioral heterogeneity.

46. The functional potential of two-component signal transduction systems

Shaunak Sen, Michael Elowitz

Two-component systems (2CS) transduce environmental stimuli into phosphate flows. In the canonical case, a histidine kinase autophosphorylates and transfers its phosphate to a response regulator protein, activating it. However, the number and configurations of domains varies widely across 2CSs, and this generates a diversity of phosphate flow architectures. Extensive research has identified phosphate flows that regulate behavior in bacteria, archaea and plants. However, the significance of this architectural diversity is unclear. Here we perform a computational analysis of the relationship between 2CS architecture and systems level function. Our results so far suggest that 2CS's provide an enhanced range and extent of sensitivity in signal transduction compared to other possible systems. Some architectures can exhibit nonmonotonic stimulus response curves.

We now seek to apply these computational insights to a natural signal transduction system in the bacterium *Bacillus subtilis*. *B. subtilis* differentiates into dormant spores when the environment inhibits growth. This process is regulated by a variant 2CS architecture called a phosphorelay, together with associated phosphatases and transcriptional feedback loops. Our earlier analysis posits how the response of this network should change due to architectural perturbations to the phosphorelay. We are now testing these hypotheses using time lapse microscopy.

47. Transcription factor dynamics in pluripotent and differentiating mouse embryonic stem cells

Frederick E. Tan, Michael Elowitz

Mouse embryonic stem cells (mESCs) can differentiate into trophectoderm, primitive endoderm and embryonic germ layers to generate any cell type found in the mouse. Within mESCs, a unique transcriptional network exists to maintain an undifferentiated and self-renewing phenotype, anchored primarily by the activity of three transcription factors: Oct4, Nanog and Sox2. The interplay between these three pluripotency regulators and specific differentiation cues (such as Cdx2, Gata-6 and T) control the transcriptional network and the dynamics of cell specification in culture. Even though direct interactions between these key lineage regulators have been identified or proposed, it is still unclear how they cooperate to enact a differentiation program and how their expression correlates with changes in cell morphology and behavior. We analyze the maintenance of pluripotency and the reinforcement of differentiation in mESCs in terms of underlying gene regulation dynamics. Experiments are based on tagging of fluorescent proteins to transcription factors of interest and tracking their expression using time-lapse fluorescence microscopy and quantitative image analysis tools. By perturbing the expression of developmental regulators and observing the downstream effect on pluripotent gene regulation, we hope to determine effective regulatory interactions and better understand the design of the transcriptional circuitry. Single-cell analysis of pluripotency and differentiation will enable us to analyze the role of variation, or noise, in these circuits. Although there are many concerns regarding the use of *in vitro* cell culture in the study of mouse development, this analysis should yield insights to further the current understanding of mammalian embryogenesis and *in vitro* manipulation of embryonic stem cells.

Mouse embryonic stem cells (MESCs) provide a unique system to study these decision-making processes. *In vitro* culture conditions allow us to understand early developmental decisions as well as later events that are technically difficult to observe *in vivo*. For example, the movements of the primitive streak lead to the formation of various mesoderm and endoderm progenitors, but in the embryo this event takes place post-implantation and single-cell imaging is intractable. Our lab is seeking culture conditions that simulate the mesoderm/endoderm transition. Using quantitative time-course antibody stains, and ultimately, fluorescent reporters for developmental master regulators, we will characterize the molecular events that underlie each decision. Are there regimes where the activity and dynamics of transcription and signaling factors commit a cell to specific decision? Or are fates indifferent to this type of cellular variability? A quantitative analysis such as this will hopefully lead to a greater understanding of MESCs differentiation and contribute to our knowledge developmental processes.

Publications

Rosenfeld, N., Perkins, T.J., Alon, U., Elowitz, M.B. and Swain, P.S. (2006) A fluctuation method to quantify *in vivo* fluorescence data. *Biophysical J.* **91**(2):759-766.

Suel, G.M., Garcia-Ojalvo, J,. Liberman, L.M. and Elowitz, M.B. (2006) An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* **440**(7083):545-550.

Süel, G.M., Kulkarni, R.P., Dworkin, J., Garcia-Ojalvo, J. and Elowitz, M.B. (2007) Tunability and noise dependence in differentiation dynamics. *Science* **315**:1716-1719.

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***Rotation student*

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Summary: Our laboratory has dedicated itself to an integrative approach to defining the cell and molecular basis of embryonic patterning, in which *in vivo* imaging tools play a central role. The explosion of data from molecular approaches and the dramatic progress from *in vitro* culture assays have resulted in a rich set of proposals for the mechanisms that underlie developmental pattering. Systems biology provides a means to organize this wealth of data, but requires some means to test the proposed linkages. Our goal is to test these proposed mechanisms in the intact embryo, with the hope of moving forward to an understanding of which of the potential mechanisms operate in the natural biological context. There are many challenges to such tests, including the tagging of cells or molecules so that they can be followed in the intact system, the visualization of the tagged structures, and the interpretation of the time-varying events these images represent. Solutions to these challenges require the coordinated efforts of researchers spanning the life and physical sciences.

In the past year, we have made significant advances in understanding the formation of the cardiovascular system in the zebrafish embryo. A major challenge in studying the development of the heart has been that key developmental events take place while the heart is beating twice a second. To answer this challenge, we have helped to refine a new microscope, now available commercially, with the ability to collect confocal laser scanning images at rates of 100's of frames per second. This permits the direct imaging of events within a normal embryonic heart as it is undergoing its dramatic morphogenesis, forming chambers and valves. New image processing tools allow us to assemble time-series data from many optical sections into a four-dimensional rendering of the moving cardiac cells together with detailed maps of the blood flow. New image analysis techniques permit quantitative analyses of these motions, and are permitting tests, in collaboration with the Gharib lab (Bioengineering, Caltech), of the roles fluid flows and forces play in the shaping of heart chambers and valves.

In parallel with the refinement of new imaging tools, we have been creating new and more efficient means

for creating embryos with genetically-encoded fluorescent tags. Through random insertion into the genome, this approach permits the creation of functional fusions between a host protein and a fluorescent protein, permitting cells and developmental questions to be posed in normally developing embryos. The creation of these new lines is dramatically more efficient than previous approaches, making it possible for even modest sized facilities to embark on large-scale screens. This increased efficiency of creating marked strains requires a parallel increase in imaging technologies, and the refinement of *in toto* image acquisition and analysis tools answers this challenge.

There have been dramatic advances in other areas as well, ranging from the refinement of new sensor technologies with researchers in the Kavli Nanoscience Institute, to improved tools for acquiring information from MRI images. Our combination of modern and classical technologies continues to offer surprises, including the discovery of a novel sensory pathway in the mammalian olfactory system and the development of a molecular sensor with unprecedented sensitivity.

48. The Digital Fish Project – Enabling systems biology in a vertebrate embryo

Sean G. Megason, Le Trinh, Niles Pierce, Scott E. Fraser, Marianne Bronner-Fraser

The genome provides a program for creating an organism. Understanding how this program works will require moving from the one-dimensional string of letters that has now been determined for the genomic code to understanding how this code is executed in the 4 dimensions of space and time of a developing embryo. Current "omic" methodologies are great for assaying across the genomic dimension but poor at assaying across space and time where the genome actually functions. We are developing imaging-based techniques that allow genome function to be probed quantitatively and systematically across the dimensions of space and time. Specifically, *in toto* imaging uses time-lapse, laser-scanning microscopy to track all the cells in a developing tissue and custom developed software to extract quantitative, cell-based data. We are using *in toto* imaging to determine complete lineages of developing tissues and to quantitate gene expression patterns. FlipTraps, a novel gene-trapping approach, allow a variety of data to be read-out *in vivo* including protein expression patterns, transcriptional expression patterns, protein subcellular localizations, and conditional mutant phenotypes. We have recently been awarded a grant from the National Human Genome Research Institute for the establishment of a Center of Excellence in Genomic Science for the "*In Toto* Genomic Analysis of Vertebrate Development." As part of this center, we plan to scale up our efforts on *in toto* imaging and FlipTraps to generate quantitative, systematic data suitable for a systems biology-based approach to development, and to integrate these data to develop a Digital Fish that recapitulates zebrafish development.

49. Fluorescent tagging of zebrafish proteins at their endogenous loci

Le A. Trinh, Sean Megason, Scott E. Fraser

Organ formation is driven by the temporal and spatial expression of specific genes during development. The long-term goal of our research is to identify and understand how genetic networks in the developing organs translation into morphogenetic processes that drive their formation. While genetic information is encoded in DNA sequence, proteins are the functional unit in most genetic pathways. Therefore, we have developed a protein-trapping vector to identify genes based on subcellular protein localization in the developing embryo and generate conditional mutant alleles by Cre-lox recombination. Using the Tol2 transposable system, we have engineered an integration vector containing citrine (YFP) flanked by splice acceptor and donor sequences in the forward orientation. In this vector, we also included lox sites and cherry (RFP) sequences in the reverse orientation. In the presence of Cre, the lox sites undergo recombination to excise the citrine and splice donor sequences and invert the cherry sequence into the forward orientation. The flipping of the cherry sequences and deletion of the splice donor allows for the generation of mutant proteins from the trap locus. In a pilot screen of 395 F_0 fish, we demonstrate that insertion of the trap vector can generate functional YFP fusions with native proteins at a frequency of approximately 1 per 10 fish screened. Genetic data indicate that the YFP protein fusions are functional as the proteins rarely generate loss-of-function phenotypes when homozygous. Additionally, we demonstrate that the fusion proteins exhibit distinct subcellular localization that is reflective of the gene function. We have identified the genes trapped by our vector through 3'RACE analyses. We are currently generating conditional mutant alleles by Cre-lox recombination to study the function of the trapped gene. The ability to generate conditional alleles will have great potential for identifying novel regulators of vertebrate development. Furthermore, the fluorescently-tagged protein traps can serve as reporters for cellular processes in many diverse applications.

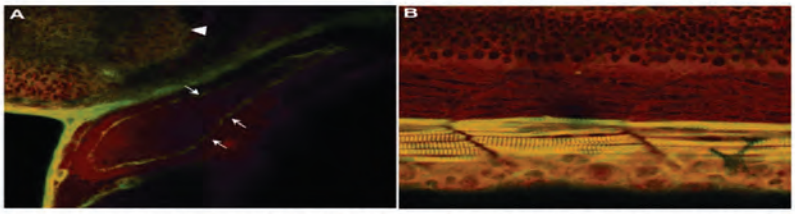


Figure legend: Confocal image of fliptrap5a (green) counterstained with the vital stain bodipy-TR methyl ester (red). (A) Cytoplasmic localization of fliptrap5a in the endocardial layer (arrows) of the heart, eye (arrowhead) and skin. (B) Z-band localization of fliptrap5a in the slow muscles fibers of the somites.

50. Analysis of Cre-recombinase induced FlipTrap mutation in zebrafish

Rebecca Barter, Sean Megason, Scott E. Fraser

The Ft8a zebrafish line was developed using the FlipTrap cassette, which was designed to form a fusion protein when it is inserted into an intronic region. This line was then injected with the cre-recombinase, which generated a mutant phenotype. When exposed to the cre-recombinase, the Ft8a line was also designed to flip the FlipTrap cassette to reveal the RFP variant with a red fluorescent tag. The FlipTrap + cre-recombinase did not reveal the RFP signature, but the mutation was evident.

The mutation phenotype included mild to severe, fatal edema associated with an enlarged heart cavity with possible malformation of the heart. The midbrain hindbrain barrier (MHB) exhibited a delay in formation for those exhibiting the full mutation phenotype. The hindbrain and midbrain also exhibited malformation, possible tissue degeneration, and possible vasculature defects.

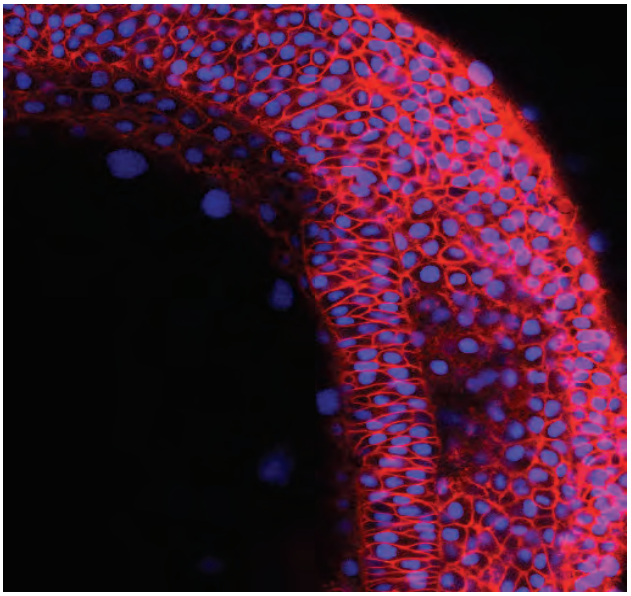
The development of the Ft8a + cre line was significantly delayed up until 70 hours past fertilization. This phenotype was characterized by measuring the angle formed between the head and trunk axes. At 70 hours past fertilization, the mutants regain normal angle. Other characteristics such as reduced pigment, edema, and MHB affects remain.

This line was out crossed and raised. Fish were randomly crossed until their clutch exhibited a 25% mutant phenotype occurrence frequency. To track this mutation, various methods of *in toto* imaging were used including fluorescent confocal microscopy of the Ft8a line. The Ft8a + cre line was also imaged with nuclear EGPF and membrane cherry to image the midbrain-hindbrain barrier (MHB) mutation, heart, vasculature, and somites.

51. *In toto* imaging of zebrafish spinal cord development

Alana Dixson, Sean Megason, Scott E. Fraser

We have injected mRNA encoding fluorescent fusion proteins to create cerulean nuclear membranes and cherry red cell membranes into one-cell stage embryos of either Top Longfin (TL) or AB wild-type stock. The translated fusion proteins allow individual resolution of each cell in the developing spinal cord during the first 24-48 hours of embryonic development. Our overall goal is to track each of the cells of the developing spinal cord from the early shield stage through at least 20 somites. We have used confocal microscopy to visualize the labeled cells and using the software GoFigure™, developed by Sean Megason, to track these cells throughout a 48-hour time series collection.



Notochord
Wild-type AB zebrafish ~1 somite
Injected at one-cell stage with:
H2B::cerulean mRNA
Membrane: m-cherry mRNA

52. Neural crest cell contribution to trigeminal ganglion development in zebrafish

Alana Dixson, Celia Shiau[*]*, Sean Megason, Marianne Bronner-Fraser, Scott E. Fraser*

We are investigating the contribution of neural crest cells to the developing trigeminal ganglion (TG) during the first 48 hours of zebrafish embryonic development. There are contributions to the TG from both the neural crest and the sensory placodes, and the details of their relative contributions and the interactions between the populations have yet to be fully defined. With antibodies to HNK1, a marker for TG cells, in a HuC::EGFP transgenic line, we have visualized the TG using a confocal microscope. Our goal is to follow the neural crest and placode cells as they combine to form the TG.

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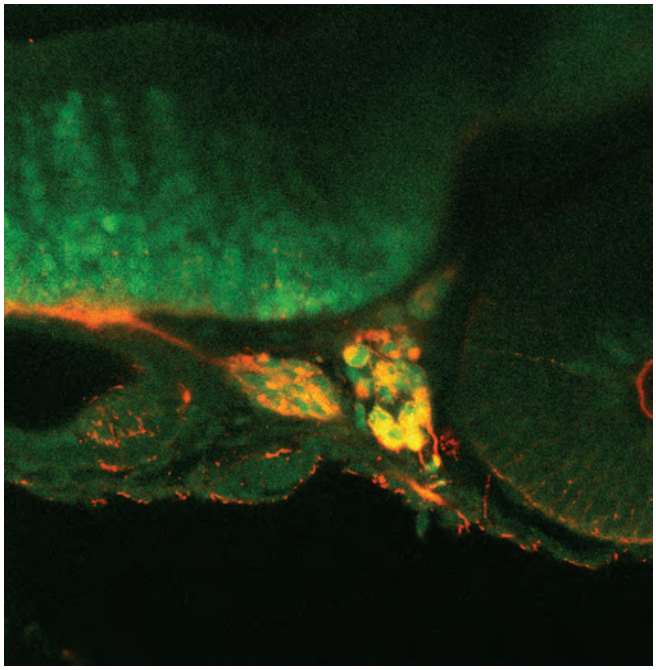

Neural crest cells in forming TG
HuC::EGFP transgenic Top Longfin zebrafish ~20 somites
1^{o} Antibody: HNK1 MAb
2^{o} Antibody: Anti-mouse Alexa 546

53. Improving microscopy to reach *in toto* imaging of zebrafish embryos

Thai Truong, Willy Supatto, Sean Megason, Scott E. Fraser

In order to reach *in toto* imaging of zebrafish embryos, the fluorescence microscopy technique requires improvement in order to reach the goals of (i) depth penetration; (ii) time/spatial resolution; (iii) multicolor imaging; and, (iv) photo-toxicity. We have been testing the setups available at the Biological Imaging Center (BIC) (confocal and 2-photon (2P) Zeiss LSM 510 microscopes) and two demo setups (the LaVision TriMScope microscope and the Coherent Micra/Silhouette laser/pulse-shaper combo), in order to precisely determine the current limitations and to formulate a strategy for the future developments.

We have found that the existing Zeiss setups of the BIC will require reworking to support of wavelengths longer than 970 nm to make them optimal as systems for *in toto* imaging. From our experience with the TrimScope demo setup, the following features are critical for the imaging setup: (i) minimal distance between sample and fluorescence light detectors; (ii) optimized optics for specific wavelengths being used; and, (iii) maximal aperture-size for light-collecting optics to capture as many fluorescence photons as possible.

We have also confirmed that fluorescence yield is inversely proportional to the laser pulse duration. However, significant complications accompany the use of shorter pulses, and we have found qualitative evidence of higher phototoxicity for shorter pulses. Hence, the standard pulse duration of ~150 femtosecond, existing in many lasers available today, should suffice for the needs of *in toto* imaging. We should, nevertheless, implement dispersion compensation to maintain the laser pulses, given an initial duration coming from the laser, at the same duration at the sample.

Given all the requirements necessary for *in toto* imaging, and that no commercial systems seem to satisfy all of these requirements, we are exploring the possibility of building an optimal imaging system ourselves. We are currently working on choosing hardware and software platforms that will give us the needed performance and flexibility of the system.

54. Neural plate and neural crest morphogenesis

Max Ezin[*]*, Scott E. Fraser, Marianne Bronner-Fraser*

The morphogenesis of the neural plate in the chick is a complex process that involves cell division, cell rearrangements and expression of genes by various cell types in specific locations. To understand the placement of the neural crest at different times, we generated a fate map of the neural plate in the epiblast of the chick. Our methods included static fate-maps and time-lapse imaging. In both cases, small populations were marked by the application of lipophilic fluorescent dyes such as DiI and DiO. These vital dyes offer cellular resolution of cell movements and cell fate in the developing embryo. We find that the neural crest is patterned along the rostrocaudal axis at early stages. Moreover, the cell rearrangements that occur rostrally in the neural tube are dramatically different from those seen caudally. Rostral rearrangements occur along the mediolateral axis of the embryo, while such rearrangements are very restricted caudally. Conversely, caudal groups of labeled cells spread along the rostrocaudal axis to a great extent, while such rostrocaudal spread is very restricted in the rostral end of the embryo. Our work will shed light on the movements of neural crest cells relative to other cell types in the neural plate.

[*]*Postdoctoral Scholar, Bronner-Fraser Lab, Division of Biology, Caltech*

55. Dynamic computational microscopy for *in vivo* imaging during embryonic heart morphogenesis

Michael Liebling, Arian S. Farouhar[1], Julien Vermot, Morteza Gharib[1,2], Mary E. Dickinson[3], Scott E. Fraser

We have developed a method for studying the functional dynamics of the developing embryonic zebrafish heart. We combined a fast confocal slit-scanning microscope, novel strategies for collecting and synchronizing cyclic image sequences to build volumes over the entire depth of the beating heart with high temporal and spatial resolution, and, finally, data analysis and reduction protocols for the systematic extraction of quantitative information to describe phenotype and function.

At the core of this method is a non-uniform synchronization problem that we solved using dynamic programming, by minimizing a criterion based on wavelet-coefficient differences. This approach has proven to be both fast, robust, and well suited for the experimental data that we collected *in vivo* on a large number of fish. We have used this approach to characterize blood flow and heart efficiency by imaging fluorescent protein-expressing blood and endocardial cells as the heart develops from a tube to a multi-chambered organ (see Figure 1). Further developments of this technique include synchronization of images from different modalities and its generalization to dynamic three-dimensional imaging of any stereotypic movements that occur in biological samples.

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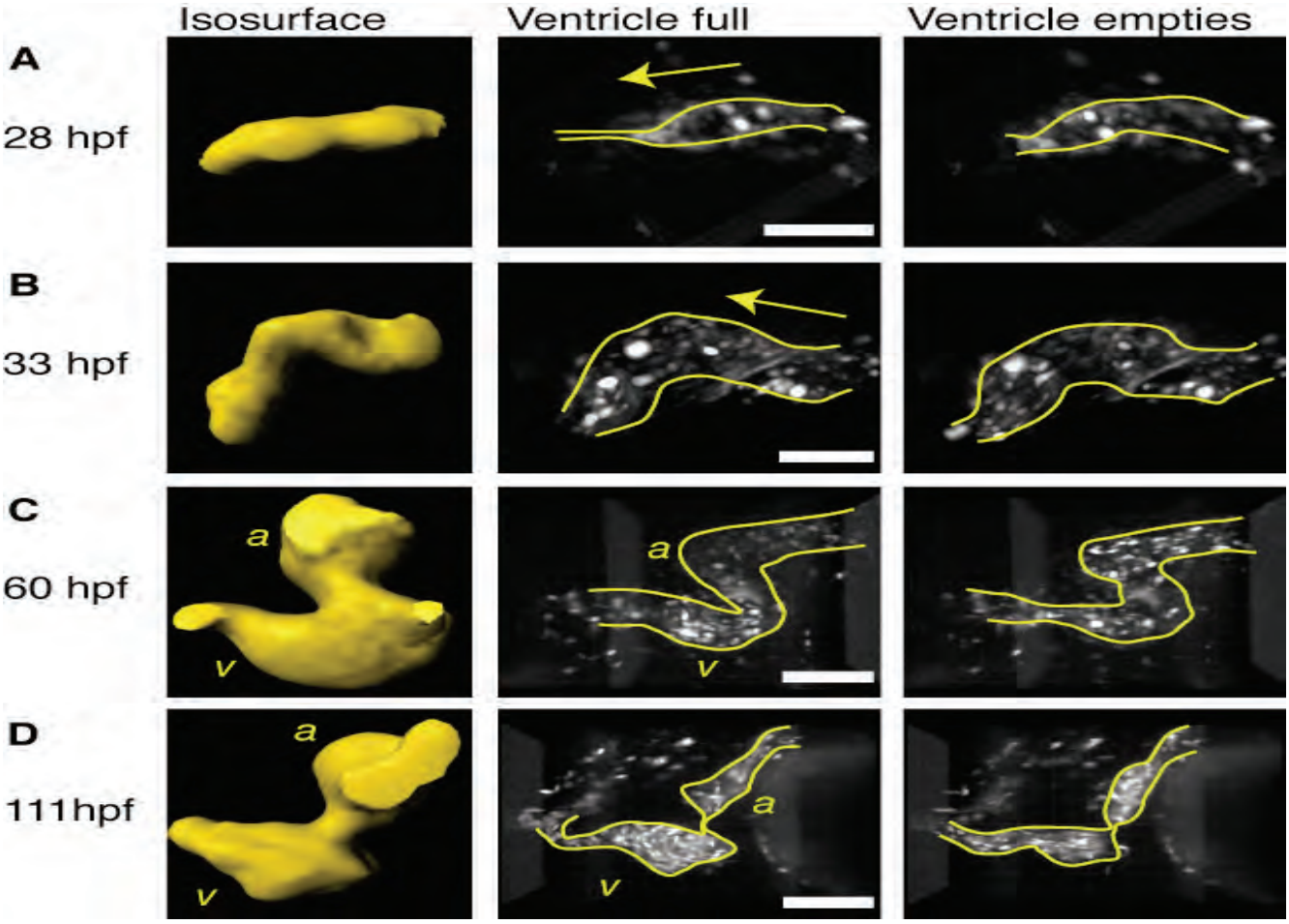

Figure 1: Dynamic three-dimensional reconstruction of the developing Tg(gata1:GFP) zebrafish heart at different stages (28, 33, 60, and 111 hours post fertilization (hpf)) at peak diastole and systole. Left column corresponds to isosurface renderings obtained during mid-diastole. These renderings of the three-dimensional architecture at a single time point allow following the morphological changes that are apparent as the heart develops from a tube (A), then loops (B) to form chambers (C) and to finally become a mature heart (D). a: atrium, v: ventricle. Arrows indicate flow direction. Scale bars are 100mm.

56. Reduced oscillatory flow induces heart valve dysgenesis

Arian Forouhar, Julien Vermot, Michael Liebling, Diane Plummer, Dannah Almasco, Morteza Gharib, Scott E. Fraser

Mature heart valves function to prevent retrograde flow between chambers of the heart. The mechanisms that govern heart valve development are not completely understood. Recent work from our lab has shown that: (i) the forces associated with blood flow through the developing heart help shape the heart; and (ii) there is significant oscillatory flow between chambers during early heart development. It is our hypothesis that oscillatory flow, a biologically active flow pattern shown to influence the expression patterns of many cardiac related genes, is a main player in heart valve development.

We have carried out a set of experiments in zebrafish that test this hypothesis by mechanically changing flow through the heart, as well as genetically compromising the heart's ability to sense shear stress. We altered the amount of oscillatory flow in the developing heart by controlling heart rate. We also decreased shear stress through the heart by decreasing contractility. In addition, we knocked down a shear stress responsive transcription factor expressed in the developing valve. In each case, heart valves displayed abnormal development and the abnormal phenotypes were conserved between the different experiments. Together, these results show the importance of oscillatory flow during heart valve development and demonstrate that relatively subtle perturbations of normal flow patterns can lead to severe valve dysgenesis.

57. *In vivo* cartoid arterial strain mapping using displacement-encoded MRI

Alex P. Lin, Morteza Gharib, Scott E. Fraser, Han Wen[*]

Atherosclerotic cardiovascular disease is the leading cause of death with over 19 million deaths worldwide where a large portion of these victims are asymptomatic. Therefore, there is a considerable demand for the early diagnosis of atherosclerosis, otherwise known as hardening or stiffening of the arterial walls. Epidemiologic studies have demonstrated that carotid artery wall stiffness contributes to systolic hypertension, increased cardiovascular risk, and risk of ischemic stroke. Furthermore, biomechanical models of the carotid artery have demonstrated increased atherogenesis in regions with high strain. We developed a displacement-encoded MRI (DENSE) sequence for imaging the motion of the carotid artery wall and mapping the 2D circumferential strain in wall. With increased resolution and regional accuracy, this technique could potentially give more efficacious risk indicators of atherosclerotic cardiovascular disease. Our aim was to validate DENSE using both *in vitro* models and *in vivo* studies. Results from *in vitro* studies demonstrate high-resolution images that accurately measure strain at several time points in the simulated cardiac cycle. *In vivo* studies were conducted on 20 healthy volunteers at both 1.5T and 3.0T MRI field strengths. Global strain in the wall of the carotid arteries measured by DENSE were compared with changes in the lumen diameter using cine MRI at the same time points and slice location. At both field strengths an excellent correlation ($R^2 = 0.58$, $p<0.05$; $R^2 = 0.86$, $p<0.005$ at 1.5T and 3.0T, respectively) therefore validating DENSE strain measurements. Older subjects showed significantly decreased strain when compared to the young subject strain measurements ($p<0.005$), as expected from epidemiological studies that have shown that strain decreases with age. Furthermore, DENSE strain measurements increased in superior slices closer to the bifurcation which corroborates with excessive stretching at the carotid bifurcation. In conclusion, DENSE measurements of strain corroborate with both clinical and biomechanical views of strain in the carotid arteries in normal controls. Future studies will demonstrate the efficacy of this technique in patients with known atherosclerosis.

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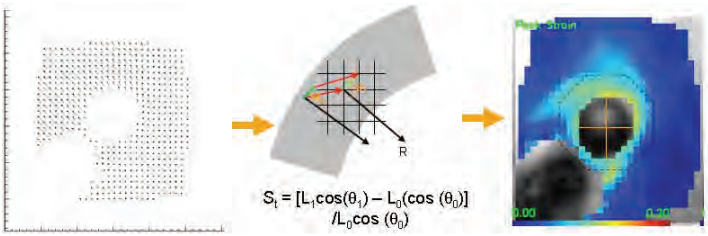

Figure 1. DENSE measurements Left: Displacement map. Middle: Strain calculation. Right: Strain map. Dotted line indicates the contour from which average strain is calculated. Orange lines indicate how CINE lumen diameters were measured.

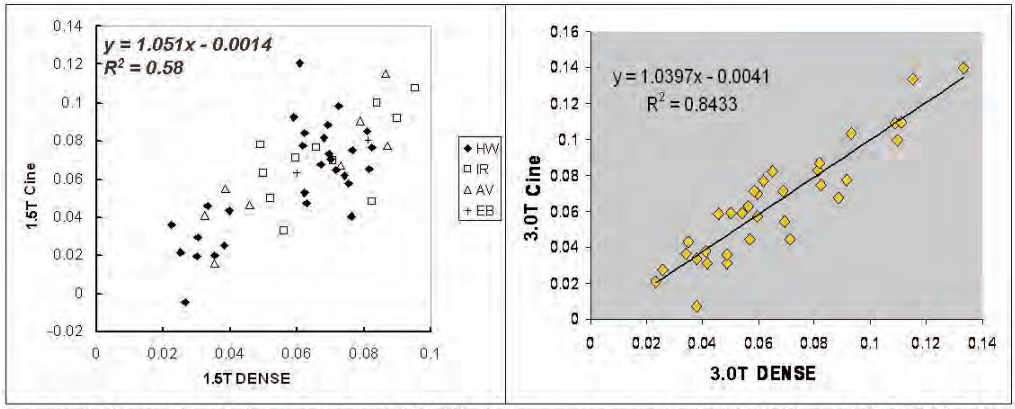

Figure 2. Correlation between cine and DENSE strain measurements at 1.5T (left) and 3.0T (right). Linear fits show excellent correlation validating the accuracy of DENSE strain measurements.

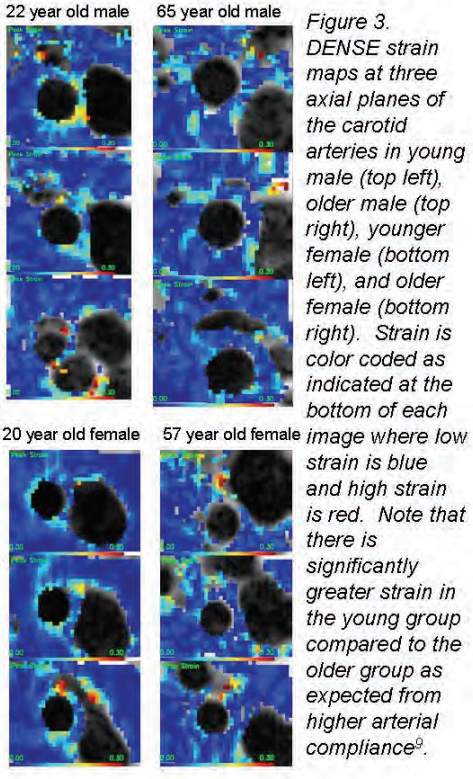

Figure 3. DENSE strain maps at three axial planes of the carotid arteries in young male (top left), older male (top right), younger female (bottom left), and older female (bottom right). Strain is color coded as indicated at the bottom of each image where low strain is blue and high strain is red. Note that there is significantly greater strain in the young group compared to the older group as expected from higher arterial compliance[9].

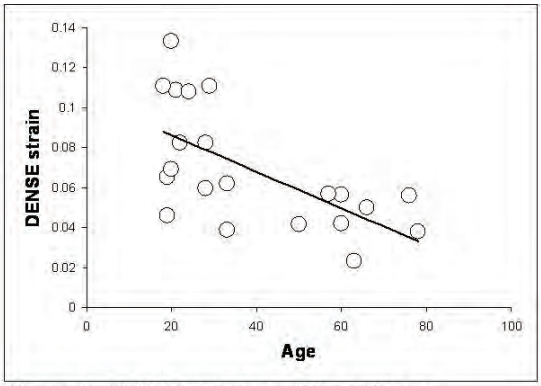

Figure 4. DENSE strain correlation with age.

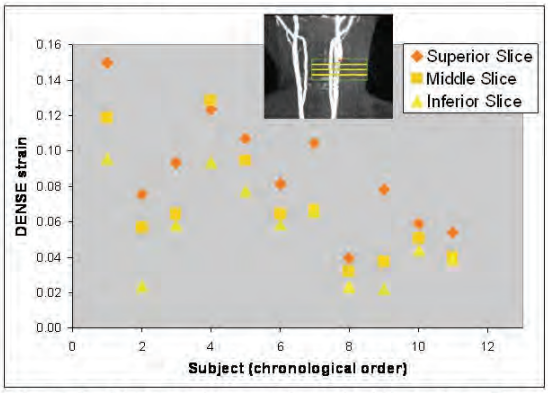

Figure 5. Characterizing strain at the bifurcation. The superior slice is closest to the bifurcation (indicated by red arrow) as shown in the image inset.

58. Mechanism of contraction in the zebrafish embryonic heart tube

Jennifer Yang, Morteza Gharib, Scott E. Fraser

In the developing zebrafish embryo, heart tube formation begins at 24 hours with rhythmic contractions following shortly thereafter. Initially, the beating heart tube was assumed to operate as a peristaltic pump with the muscle contraction beginning at one end and propagating the length of the tube as a wave. Previous research in this lab, using technological advancements in confocal microscopy to reconstruct four-dimensional images of the embryonic heart tube, has shown discrepancies with the peristaltic pump model. Newly acquired images of the beating heart tube reveal a bi-directional wave propagating from the site of contraction, backflow of blood within the system, and blood cell velocities exceeding the speed of the contractile wave present in the heart tube. Contrasting with the original model of a peristaltic pump, these recent discoveries indicate that the embryonic heart tube behaves like an impedance pump.

The impedance pump works by creating a contraction off-center on an elastic tube, which then generates a bi-directional wave that produces suction within the tube to move the majority of blood cells in a forward direction. If this model accurately describes the mechanics of the zebrafish embryonic heart tube, then we should find a localized contractile ring to initiate wave propagation to circulate blood through the vasculature. Contraction of cardiomyocytes involves an influx of calcium ions into the cell. Troponin binds the calcium ions, causing the muscle fiber to undergo a conformational change, which shifts the position of tropomyosin. This process reveals the binding sites on the actin filaments that attach to myosin and produce the muscle contraction. With the use of a calcium indicator, the muscle cell will fluoresce when calcium ions enter the cell just prior to binding troponin. Through high-speed ratiometric confocal imaging, the fluorescent contractile cardiomyocytes can be identified separately from the cardiomyocytes of passive contraction, thus allowing for determination of the precise pumping mechanism in the embryonic heart tube.

Reference

Forouhar, A.S., Liebling, M., Hickerson, A., Nasiraei-Moghaddam, A., Tsai, H.J., Hove, J.R., Fraser, S.E., Dickinson, M.E. and Gharib, M. (2006) *Science* **312**:751-753.

59. Asymmetric flow and left-right axis establishment in zebrafish

Julien Vermot, Michael Liebling, Willy Supatto, David Wu, Scott E. Fraser

Cilia dynamic works in many, if not all, vertebrates to set and maintain the left right embryonic axis. In mouse, cilia rotation triggers a unilateral flow and propels instructing factors asymmetrically within the so-called embryonic node. Several models have been proposed to explain how cilia dynamic influences this process in mouse. Although it is tempting to generalize, very few data are available to actually validate that cilia properties are conserved in other vertebrates. To clarify this issue, we characterized the beating dynamic of the cilia and the resulting flow using state of the art imaging tools in zebrafish. Our approach uses different types of imaging, from bright field imaging, fast confocal microscopy to multiphoton imaging. These observations led us to propose an alternative hypothesis that we are currently testing using the molecular tools available in zebrafish. Our results currently suggest that cilia beating properties diverge between mouse and zebrafish and raise the possibility that cilia have been co-opted in vertebrates to fulfill different propelling roles in the left-right cascade due to hydrodynamic reasons.

60. Analytical modeling of hydrodynamics of the Kupffer vesicle

David Wu, Julien Vermot, Jon Freund[*], Scott E. Fraser

We seek to elucidate the mechanism for determination of left/right asymmetry in the adult body plan of zebrafish. One of the posited early mechanisms for left/right determination is the existence of a ventral structure – in zebrafish, named Kupffer's vesicle (KV) – that transports fluid uni-directionally. It usually consists of a small, depressed area (~100 mm^2) lined with beating cilia which creates a field of flow: the putative mechanism(s) for symmetry breaking could be the transport of released packets of information from one side of the embryo to another; the generation of morphogen gradients within the convexity; or merely from a mechanical signal induced by the field of fluid flow. We seek to quantify and map the flow vectors of the circulating fluid at multiple points simultaneously through a combination of modeling and experiments. Modeling consists of implementing a Green function for Stokes flow in a sphere with force transducers given by the placement of cilia, emulating the low Reynold's number environment inside the KV. Experiments consist of trapping endogenous (or exogenous) objects using a laser tweezer and then releasing the particles over different areas of the KV and tracking the resulting movement. Preliminary results indicate the existence of a differential shear between the (undefined) left and right axes.

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61. A multi-photon multi-dimensional analysis of *Drosophila melanogaster* mesoderm formation

Willy Supatto, Amy McMahon, Angelike Stathopoulos[1], Chin-Lin Guo[2], Norbert Perrimon[3], Eric Brouzes[3], Scott E. Fraser

An embryo is shaped by a fascinating and complex choreography of coordinated cell movements that are highly regulated in time and space. The first morphogenetic movement during *Drosophila* embryo development is the invagination of the presumptive mesoderm, taking place at gastrulation. At this early developmental stage, the structure of the embryo is

relatively simple, making this event both a tractable model of invagination and a beautiful example of cell collective behavior.

Although the genetic control of mesoderm invagination has been extensively studied, many questions remain to be elucidated, including: How are cell shape changes triggered and coordinated in order to make an invagination? Do external mechanical forces play a role in this process? What is the part of active and passive mechanical behavior? What is the nature of cell-cell interaction involved in this collective process: biochemical, mechanical or both? What is the spatio-temporal scale of this interaction?

Drosophila embryo is an outstanding model for studying the genetic control of morphogenesis. However, its physical properties make it challenging to image and investigate its biomechanics *in vivo*, compared with other models. Early *Drosophila* embryos develop quickly and are highly scattering for visible light. As a consequence, conventional confocal fluorescence microscopy is limited to less than a third of the required imaging depth, and typically induces strong photo-bleaching/photo-toxicity. In addition, the vitelline membrane surrounding the embryo prevents the investigation of cell biomechanics when using invasive methods. As a result, each of the above questions, which are central to our understanding, cannot be fully studied by conventional imaging/manipulation tools. Our goal is to circumvent these limitations by using new tools and create a complete study of tissue dynamics *in vivo* in the important system of *Drosophila* embryo mesoderm invagination. In collaboration with several groups at Caltech and at Harvard Medical School, we are developing the following research program:

(i) 4D imaging of mesoderm invagination by using advanced nonlinear microscopy that is adapted for the 4D, long-term and deep tissue imaging of *Drosophila* embryos without compromising viability.
(ii) Systematic quantification of morphogenetic events by using advanced image processing/analysis techniques.
(iii) Genetics and physical disruption to elucidate the mechanical and biochemical relationship involved in the coordination of movements.
(iv) Together these experimental data are integrated in a model to help our understanding of the collective process of invagination.

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[3]*Genetics Department, Harvard Medical School*

62. Investigating the role of the Grueneberg ganglion in mammalian olfaction

David S. Koos, Scott E. Fraser

The Grueneberg ganglion is a small nerve located in the lining of the far rostral mammalian nasal vestibule. Historically, this nerve was thought to be a component of the terminal nerve, a non-sensory neuroendocrine nerve that does not innervate the first relay station of the primary olfactory pathway, the olfactory bulb. After noting that the Grueneberg ganglion expresses the pan-olfactory marker Olfactory Marker Protein (OMP), we suspected that this ganglion might instead be part of the olfactory pathway. Through the use of axon tract tracing techniques we have demonstrated that the Grueneberg ganglion projects its axons to a little known specialized sub-domain of the olfactory bulb, referred to as the olfactory necklace glomeruli. The expression of OMP combined with its direct wiring to the olfactory bulb suggests that the Gruenberg ganglion is a previously unrecognized component of the primary olfactory pathway in mammals. Our current work is focused on elucidating the axonal behaviors and molecular coding that underlie the connectivity of this ganglion with the necklace glomeruli. These experiments will provide important insight into the molecular nature and potential chemosensory function of this unusual olfactory nerve.

Reference

Koos, D.S. and Fraser, S.E. (2005) *NeuroReport* **16**:1929-1932.

63. Prominent fixative-induced fluorescence in the rodent Grueneberg ganglion

Cambrian Y. Liu, David S. Koos, Scott E. Fraser

The Grueneberg ganglion (GG) is a clustered, bilateral collection of ~1000 neurons whose somata are located in the septal corners of the murine cartilaginous nasal vestibule. Neurons of the GG express olfactory marker protein and project axons innervating glomeruli of the necklace domain of the olfactory bulb. However, a chemosensory function has not been assigned to the GG. Unlike other primary sensory cells of the mouse olfactory system, the cells of the GG cannot access the nasal cavity where potential odorants are located. The functions of the GG have been proposed to be either vestigial or invoked only in atypical situations (e.g., suckling). Our work with the GG in adult mice has revealed that this ganglion possesses an intense broad-spectrum autofluorescent signal when mouse tissue is fixed for 48 hours either with heat-treated paraformaldehyde or a glutaraldehyde/ paraformaldehyde mix. The fixative-induced fluorescent signal was evenly distributed throughout the GG cell somata and was water-stable. Because fixative-induced fluorescence typically reveals biogenic amines, our observations suggest that the GG constantly produces biogenic amines and uses them as neurotransmitters well into adulthood. The intensity of the fluorescence in the somata suggests that the GG is constantly producing new biogenic amines, presumably to replace activity-depleted pools. We argue that the GG activity is commonly evoked and not restricted to atypical situations.

64. The potential and characteristics of mammalian germ stem cells

Carol Readhead, Sheba Jarvis[1], Scott E. Fraser

Germ cells maintain their totipotency throughout the life of the animal but they only realize their full potential when they become haploid gametes and fuse with another gamete to form a zygote. Amazingly, it has now been shown that diploid male germ stem cells can transdifferentiate into embryonic stem cells (ES) *in vitro*. This has led to a renewed interest in these cells as a possible source of stem cells that could be used for therapy.

A key gene indicative of pluripotency is Oct 4, a transcription factor. We have used a transgenic mouse, which expresses green fluorescent protein (GFP) under the control of the Oct 4 promoter (a kind gift from Dr. Jeff Mann, City of Hope) to isolate germ stem cells from embryonic and neonatal males. In consultation with Rochelle Diamond in the Cell Sorting Facility we have shown that these germ stem cell populations are not homogeneous and that they can be subdivided into GFP+ cells that also express c-Kit and/or Thy1.2. There are also GFP+ cells, which do not express either c-Kit or Thy1.2. The ratio of these different populations changes dramatically throughout embryonic and neonatal development and is also different from the adult population. Recently, with Rochelle Diamond's help, we have been able to sort 40,000 GFP+ germ cells from the genital ridges of E11.5 embryos. These subpopulations will be characterized using microarrays. In addition, the response of the sorted cells will be tested in different environments *in vivo*: a different species- the chick embryo, ectopic tissue - the kidney capsule, and a normal environment - the neonatal testis. Work on the chick will be done in collaboration with Dr. Bronner-Fraser. Prior to transplantation the cells will be infected with a lentiviral vector containing the fluorescent protein gene cherry under the control of the house keeping promoter, pgk (in collaboration with Rusty Lansford). If the transplanted cells start to differentiate in the new microenvironment they will loose GFP expression but will maintain the cherry.

Live confocal imaging of the GFP+ male germ cells within the seminiferous tubules shows that their behavior changes during development. After migration to the genital ridge the germ cells remain fairly static during the embryonic period and then become active on the day of birth, positioning themselves on the inner surface of the tubule. At P4 numerous germ cells undergo apoptosis. This was confirmed by histochemistry using an antibody to caspase.

[*]*Imperial College, Hammersmith Hospital Campus, London W12 ONN.*

65. Studying cell properties and development in zebrafish embryos using magnetic bead micro manipulation

Luca Caneparo, Stephanie Johnson, Scott E. Fraser

During animal development a single cell is able to give rise to different cell types and tissues. One mechanism repeatedly used to generate cell diversity is the asymmetric distribution of determinants such as proteins or mRNAs. For instance, localization of β-catenin soon after fertilization is required to position correctly the embryonic axis in both amphibians and fish. This event occurs as early as zygote period, where streams of cytoplasm directed towards the animal pole cause segregation of the blastodisc from the clearer yolk granule-rich vegetal cytoplasm. A description of the cytoplasmic properties in the developing embryo and the possibility of directly manipulating determinants is not available at the moment mainly due to the lack of micromanipulation tools. Using permanent magnets we develop a way to manipulate functionalized magnetic beads in the developing embryo. We were able to functionalize the magnetic beads using surface chemistry and decorate them with fluorescent proteins to subsequently reposition them in the blastomeres. We used this technology to gather information about cytoplasmic viscosity and to develop a method to study determinants localization *in vivo*. We therefore test different molecules that have been shown to affect early embryonic development. These results open the possibilities to study early determinants *in vivo* using magnetic beads to carry different determinants with the possibility to spatially control them during early embryos.

66. A magnetic resonance stage microscope for developmental biology

Andrey Demyanenko[1], Yun Kee[2], J. Michael Tyszka

Many popular models of early vertebrate development, including frog, lamprey and axolotl, have opaque embryos, making optical microscopy of deeper cell layers extremely difficult if not impossible. Magnetic resonance microscopy (MRM) is a non-invasive imaging technique unhindered by the optical opacity of a tissue and has recently been applied successfully to time-lapse imaging of early development in frog embryos. However, MRM current hardware limits throughput to only one or two embryos per experiment, which may last for several days. We originally proposed the development of a new magnetic resonance microscope design analogous to an inverted stage microscope that addresses these limitations and specifically targets applications in developmental biology. A functional MR stage microscope could be applied to any small, hydrated, opaque system, including embryos, insects, marine animals, biofilms, bioreactors, cell cultures, porous media and plants. The stage microscope format provides unhindered access to the sample from above, encouraging the future integration of optical imaging equipment with MR microscopy. Simultaneous optical imaging allows correlation of

superficial cell movements with deeper tissue organization from MRM. The stage microscope format is also applicable to larger organisms where MRM of superficial structures is of interest. The current uniplanar gradient coil design is highly scalable, independent of magnetic field strength and could ultimately be adapted for human imaging in larger bore, lower field MRI systems. Ultimately, the proposed instrumentation could be commercialized as an after-market insert for existing horizontal bore magnetic resonance systems. Initial data from the first prototype MR stage microscope (Figure 1) is very encouraging and demonstrates the immediate utility for non-invasive asymmetric imaging on the scale of developing frog embryos. Other application areas will be explored in the coming year, including high-resolution MR diffusion histology of brain samples and live marine animals in collaboration with the Long Beach Aquarium of the Pacific.

[1]*Assistant Scientist, Fraser lab, Division of Biology, Caltech*
[2]*Bronner-Fraser Lab, Division of Biology, Caltech*

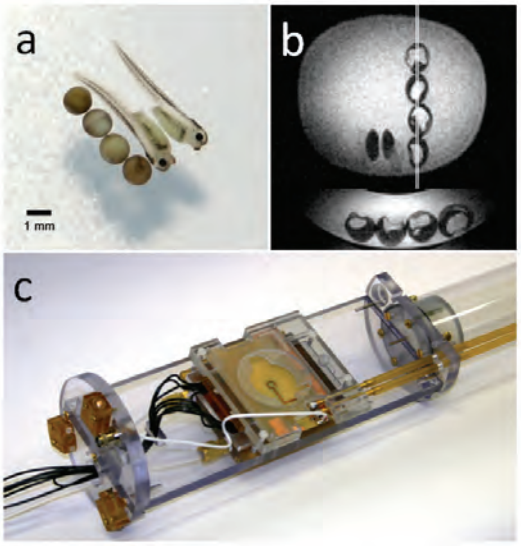

Figure 1: "First-light" on the prototype MR stage microscope. (a) Fixed *Xenopus laevis* embryos mounted in agarose for high-resolution MR histology. (b) First biological imaging performed on the stage microscope at 7 Telsa – transverse and sagittal slices through the 3D imaging volume are shown. In plane resolution is approximately 80 microns. (c) The fully assembled prototype MR stage microscope. The stage is designed to fit within the bore of conventional high-field MR imaging systems and is powered by available system RF and gradient electronics. The three-axis multi-layer gradient set is water cooled and lies beneath the RF coil visible at the center of the instrument.

67. Developing diagnostics and therapies for age-related macular degeneration (AMD)

C.J. Yu, Jeff Fingler, Jon Williams, J. Michael Tyszka

Age-related macular degeneration causes vision loss for approximately 10 million people in the United States, a number that will continue to increase over time as the population over 50 years of age increases. Severe vision loss caused by this disease is primarily caused by the occurrence of sub-retinal neovascularization, which leaks and damages the retina. Many therapies are directed towards treating this portion of the disease, halting the progression of the new vascular growth.

To increase the effectiveness of vascular inhibitor treatments, the earliest stages of vascular growth needs to be visualized. Optical coherence tomography (OCT) is an instrument currently used to optically image 3D structure of the retina of patients. Through the addition of motion contrast capabilities to OCT within this project, the imaging systems are able to visualize 3D regions of mobility and flow such as neovascularization.

Developments are underway to incorporate the motion contrast visualization capabilities into commercial imaging systems to diagnose vascularization at earlier time points than possible with current clinical technology.

Lipid accumulation between the retina and the ocular blood supply occurs over time within patients before neovascularization occurs, causing a reduction of the hydraulic conductivity and macromolecular permeability across this region. Magnetic resonance imaging is uniquely sensitive to the diffusion and transport of water in tissues. We have developed a unique MRI-compatible apparatus for estimating water diffusional permeability in *ex vivo* Bruch's membrane explants (kindly provided by Marco Zarbin and Ilene Sugino of the New Jersey Medical School). Comparing equilibration times

for matched explants pairs in pilot studies, we have demonstrated an approximately 15% improvement in water transport associated with detergent treatment of the membrane, laying the groundwork for future development of targeted therapies for AMD.

68. Digital three-dimensional atlas of quail development using high-resolution MRI

Seth W. Ruffins, Melanie Martin, Lindsey Keough, Salina Truong, Scott E. Fraser, Russell E. Jacobs, Rusty Lansford

We present an archetypal set of three-dimensional digital atlases of the quail embryo based on microscopic magnetic resonance imaging (αMRI). The atlases are composed of three modules: (1) images of fixed *ex ovo* quail ranging in age from embryonic day 5 to 10 (e05 to e10); (2) a coarsely delineated anatomical atlas of the αMRI data,; and (3) an organ system-based hierarchical graph linked to the anatomical delineations. The atlas is designed to be accessed using SHIVA, a free Java application available. The atlas is extensible and can contain other types of information including anatomical, physiological and functional descriptors. It can also be linked to online resources and references. This digital atlas provides a framework to place various data types, such as gene expression and cell migration data, within the normal three-dimensional anatomy of the developing quail embryo. This provides a method for the analysis and examination of the spatial relationships among the different the types of information within the context of the entire embryo.

We are collaborating with curators at the California Science Center and the San Francisco Exploratorium to design and develop interactive exhibits that will bring the QDA to a broader lay audience and around which we intend to develop more formal educational programming. The QDA project has been greatly advanced with high school students helping to annotate the MRI data sets using Amira software and to produce exhibit quality movies using Final Cut Pro movie editing software. We are updating on a weekly basis and a website has been established to disseminate the QDA (http://atlasserv.caltech.edu/Quail/Start_Quail.html). Our use of high school students also provides several young potential scientists with their first exposure to 'cutting edge' research and direct mentoring from research scientists.

69. Visualization of blood vessel formation in developing quail embryos

Yuki Sato, Scott E. Fraser, Rusty Lansford

The vascular system is established early during development to circulate oxygen and nutrients in the developing body. Blood vessel endothelial cells in early vertebrate embryos arise from mesodermal tissues. It remains unexplored how these endothelial precursor cells of mesodermal origin contribute to the formation of primitive blood vessels in developing embryos. Avian embryos have been good model animals to study blood vessel formation because of the easy accessibility to developing vasculature. They also enable us to perform experimental analyses, for example, surgical manipulations and DNA electroporation for studying tissue interactions and molecular mechanisms, respectively. Moreover, developmental processes of early avian embryos can be observed by using either *in ovo* imaging tools or *ex ovo* culture techniques. Despite these great advantages, vital labeling of the forming vasculature has been difficult in avian embryos. In order to understand the process of blood vessel patterning, we have been trying to visualize both forming blood vessels and migrating precursor cells using transgenic quail embryos. To label the forming vasculature and the precursor cells, we have been constructing new reporter constructs based upon the mouse VE-cadherin promoter (gift from Dr. L. Naldini) and a synthetic Notch-responsive sequence (gift from Dr. H. Okano), respectively. They were cloned into lentiviral vectors with fluorescent reporter genes so that they can be transduced into the quail genome by simple injection of the lentivirus particles at early developmental stages. While successful, these tissue-specific promoter activities were too weak to express fluorescent reporters at a level sufficient for dynamic imaging. To increase expression levels of the reporter genes, we used a Gal4VP16-UAS system to drive a higher level of expression. This system is known to enhance expression of the fluorescent reporter genes downstream of the UAS sequence because of strong transactivation by VP16 domain (Köster and Fraser, 2001). By modifying the Gal4VP16-UAS system for use in lentivirus-mediated gene transfer, we achieved expression of the reporters six-fold brighter than the conventional vector in quail embryonic cells. We are now using this to produce chimeric transgenic founder (G_0) quails carrying the GAL4VP16/UAS system. These transgenic quails are expected to be applied to tissue-specific gene overexpression experiments by combining with electroporation of a plasmid carrying the UAS-gene of interest.

70. Single particle tracking of virion vectors

Christie A. Canaria, Scott E. Fraser, Rusty Lansford

Viruses are sub-microscopic particles with the ability to infect a wide spectrum of living organisms. Different classes of viruses exist, and in general, their make-up includes genetic material of either DNA or RNA encapsulated in a protective protein coat. The efficiency by which viruses infect cells and transfer their genetic material may cause disease and damage to the hosts. However, only a small percentage of viruses are pathogenic. Understanding the pathway for viral infection and gene transfer into host cells is essential for generating effective treatments after viral infection. In addition, knowledge of viral infection pathways may lead to better virion-derived vectors for vehicles of gene therapy. We are using real-time fluorescence imaging to track and investigate the motion of single lentiviral and retroviral particles as they interact with mammalian cells. This

approach allows us to follow individual viruses and determine the intracellular pathways that lead to efficient infection. These experiments will offer both better understanding of the normal viral pathways and better designs for transgenic vectors.

71. Label-free, single-molecule detection with optical microcavities

Andrea M. Armani, Rajan P. Kulkarni, Scott E. Fraser, Richard C. Flagan[1,2], Kerry J. Vahala[2]

Single-molecule fluorescence experiments have improved our understanding of many fundamental biological processes. These breakthrough experiments required labeling of the target molecule. This restricts an experiment's scope, because there must be prior knowledge of the target's presence and the target molecule must be modified to incorporate the label. There have been several attempts to overcome this need to label the analyte by developing label-free sensing technologies, ranging from fiber-optic waveguides and nanowires to nanoparticle probes and mechanical cantilevers, but none has achieved single-molecule sensitivity. We have developed a highly specific and sensitive optical sensor based on an ultrahigh quality (Q) factor ($Q>10^8$) whispering-gallery microcavity. The silica surface is functionalized to bind the target molecule; binding is detected by a resonant wavelength shift. Single-molecule detection is confirmed by observation of single-molecule binding events that shift the resonant frequency, as well as by the statistics for these shifts over many binding events. These shifts result from a thermo-optic mechanism. Additionally, label-free, single-molecule detection of interleukin-2 was demonstrated in serum. These experiments demonstrate a dynamic range of 10^{12} in concentration, establishing the microcavity as a sensitive and versatile detector.

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72. Quantitative *in vivo* imaging of the dynamics of gene regulatory networks

Mat Barnet, Eric H. Davidson[*], Scott E. Fraser

The fundamental question of developmental biology is how a single fertilized egg cell gives rise to a complex organism containing thousands, millions, or billions of highly integrated cells. As large-scale genome projects continue to reveal the striking genetic similarities among different species, it is becoming more and more clear that interspecies variation results not from major differences in the sets of genes different organisms possess, but from differences in the regulation and expression of those genes. To better understand how gene regulatory networks govern embryonic development, we are collaborating with Eric Davidson's lab, using sea urchins as a model system. The size, shape, and optical transparency of sea urchin embryos makes them ideal for study by light microscopy. We are developing fluorescence imaging tools and techniques to enable *in vivo* quantification of the dynamics of the gene regulatory network responsible for specification of the sea urchin endoderm and mesoderm. One technique involves quantitatively imaging expression of a reporter gene (e.g., green fluorescent protein (GFP) in the three-dimensional embryo using confocal laser scanning microscopy (CLSM) or two-photon laser scanning microscopy (TPLSM). By quantifying gene expression patterns in three dimensions over time, we aim to better understand how the dynamics of gene regulatory networks govern developmental processes. Another technique involves fusion proteins consisting of a photoactivatable or photoconvertible protein fused to a protein whose dynamics are of interest, such as a transcription factor. Such fusions allow for *in vivo* pulse-chase type experiments, in which we aim to quantify protein production and turnover dynamics. By our quantitative approaches, we hope to generate kinetic data that can be applied to the current, largely qualitative, gene network models, to enable the models to make quantitative predictions of regulatory dynamics, thereby helping to further our understanding of how complex organisms develop from single cells.

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73. Synthetic *cis*-regulatory modules

Roee Amit, Scott E. Fraser, Frances Arnold[*]

Understanding the structure and function of *Cis* Regulatory Modules (CRMs) is imperative to our continuous effort to decipher the genomic code. These non-protein coding regions control when (stage of development or cell/life cycle), where (which cells), and how much (intensity of expression) a particular gene is expressed in an organism. Recent works have argued that the regulatory process controlled by CRMs is, in fact, a computational algorithm that is hard-wired into the genome[*]. It is the purpose of this project to examine this question from a constructionist approach. Namely, construct synthetic regulatory regions that will attempt to mimic some of the logic that is presumed to exist.

To that end, a model has been initially constructed. The model stipulates that logical features associated with CRM logic (expression amplification, discrete expression states, presence of multiple binding sites of a particular transcription factor in a given module, etc.) are in part due to the ability of DNA binding protein to alter the bending rigidity of DNA. This characteristic, in turn, can modify the time scale that is associated with transcription initiation by decreasing or increasing the probability of the driver activator from contacting the RNA polymerase through the process of looping, thus controlling the amount of total transcripts that are transcribed.

This model will be tested initially on a bacterial platform that is eukarotic-like (i.e., having a regulatory region containing several transcription factor binding sites upstream of the basal promoter). The goal will be generate expression-level patterns reminiscent of CRM controlled genes that can be predicted through the above looping-bending rigidity model. This will be done by designing *ab-initio* spacer regions containing binding sites for DNA binding proteins in some distribution, whose level in the

cell can be controlled by external means (i.e., LuxR, TraR). The distribution and number of binding sites will be altered using a directed-evolution type of approach, until a desired feature will be obtained (in this case, well-spaced discrete expression states). The second part of the project, will involve implementing this solution in a eukaryotic organism (either yeast, or pre-gastrulation zebra-fish embryo) and observing whether or not a similar behavior is obtained.

[*]*Professor of Chemical Engineering and Biochemistry, Caltech*

Reference

Davidson, E.H. (2006) The Regulatory Genome, Elsevier, Press, San Diego, CA

74. Effects of isoflurane on Mn^{2+} neuronal uptake and trans-synaptic transport

Xiaowei Zhang, Russell. E. Jacobs

In vivo, trans-synaptic tract tracing utilizing manganese-enhanced magnetic resonance imaging (MEMRI) has attracted growing attention among researchers in neuroscience (1). Previous work has shown that Mn^{2+} can be taken up through voltage-dependent calcium channels (VDCCs), which are ubiquitous in neurons. The increase of presynaptic Ca^{2+} on membrane depolarization is caused by entry of Ca^{2+} from the extracellular space after the opening of presynaptic VDCCs and the release of Ca^{2+} from intracellular stores. Therefore, Mn^{2+} accumulates in neurons through VDCCs in an activity-dependent manner. Based upon this fundamental principle, Mn^{2+} has long been used in biomedical research as an indicator of Ca^{2+} influx in conjunction with fluorescent microscopy. Studies on the mechanisms of the general anesthesia isoflurane indicate that it may depress Ca^{2+} entry through the presynaptic VDCCs (2). The aim of the present work is to investigate whether Mn^{2+} neuronal uptake and trans-synaptic transport are affected following long-term isoflurane inhalation.

Ten male mice were injected with 10 nL of 100 mM $MnCl_2$ in the right motor cortex and MR images were acquired 30 min and 8 hr after $MnCl_2$ injection. The mice were divided into two groups: 1) control (recovered after first MRI); and, 2) unconscious (maintained with isoflurane inhalation until the termination of the experiment). Data from two groups was entered into a general linear model fixed-effect group analysis framework using statistical parametric mapping (SPM). Both diffuse and spatially distinct enhancements are seen in the motor cortex and hippocampal areas (Fig.1). No significant differences were found between control and unconscious animals on SPM test and color-labeled projections. The VDCC family has been classified into at least T, L, N, P, Q, and R subtypes. Although involvement of individual Ca^{2+} channel subtypes in the effects of volatile anesthetics on Ca^{2+} entry is still uncertain, P-type channels are relatively insensitive to isoflurane (3), implying that Mn^{2+} is likely taken up and synaptically transported via un-inhibited VDCCs.

References

1) Pautler, R., Mongeau, R. and Jacobs, R. (2003) *MRM* **50**:33-39.
2) Kameyama, K., Aono, K. and Kitamura, K. (1999) *Br. J. Anaesth.* **82**(3):402.
3) Hall, A.C., Lieb, W.R. and Franks, N.P. (1994) *Anesthesiology* **81**:17-23.

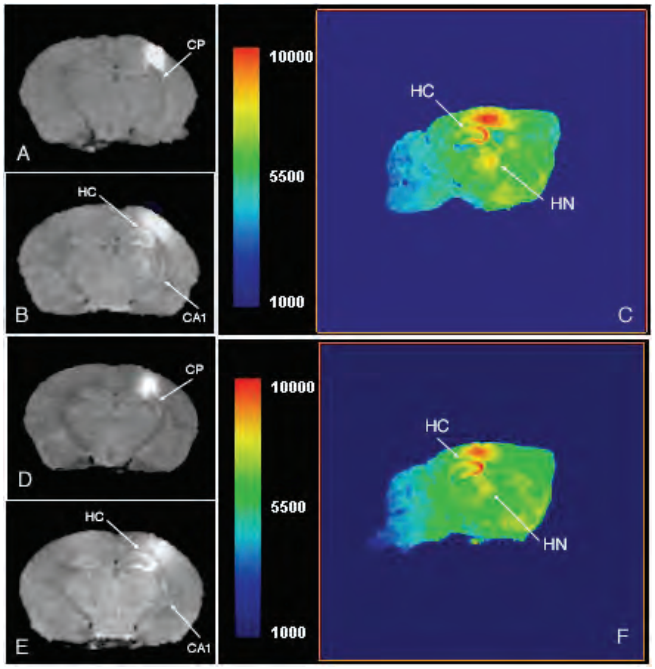


Figure 1. 3D UFLARE MRI of the mouse brain 30 minutes and 8 hours. The images (A-C) are the unconscious animal and the images (D-F) are the control animal. Note the identical color labeled projections on the sagittal views of MEMRI in these two animals. CP, choroid plexus; HN, hypothalamic nucleus; HC, hippocampus.

75. Prediction and prevention of encephalopathy in a mouse model of glutaric acidemia

Jelena Lazovic, William J. Zinnanti, Keith C. Cheng, Russell E. Jacobs

Glutaric acidemia Type I (GA-1) is a genetic disorder that interrupts a common branch of lysine and tryptophan catabolism through glutaryl-coenzyme-A dehydrogenase (GCDH). Children with this disorder may experience irreversible striatal injury within the first three years of life. The events leading to striatal necrosis are unclear and lack of an early diagnostic marker heralding brain injury has impeded intervention efforts. Dietary supplementation of *Gcdh-/-* knockout mice with lysine provides a model of acute striatal injury. We used this model to determine if changes in brain metabolite ratios, observed non-invasively using proton magnetic resonance spectroscopy (MRS), can predict irreversible striatal injury. The ability of lysine to trigger acute neuropathology in this model inspired a potential treatment strategy aimed at blocking brain lysine uptake with

homoarginine, which is taken up by the same transporter. Animals placed on 4.7% dietary lysine had ~40% decrease in the glutamate/glutamine to creatine ratio (Glx/Cr) and a ~20% decrease in N-acetylaspartate to creatine ratio (NAA/Cr) at 48 h following the diet onset. At this time point, no signs of striatal injury were detectable by T2-weighted MRI (Figure 1B). The striatal injury became evident after 114 h (Figure 1C). There were no significant MRI or metabolic changes among animals on normal diet (Figure 1A) or among animals on 4.7% lysine diet also supplemented with homoarginine, or supplemented with both homoarginine and glucose (Figure 1D, E and F). These findings suggest MRS as potentially useful diagnostic marker of impending brain injury in GA-1. Our MRI and MRS data provide supporting evidence that combined homoarginine/glucose therapy may be protective in GA-1.

Reference

Zinnanti, W.J., Lazovic, J., *et al.* (2006) *Brain* **129**:(Pt 4):899-910.

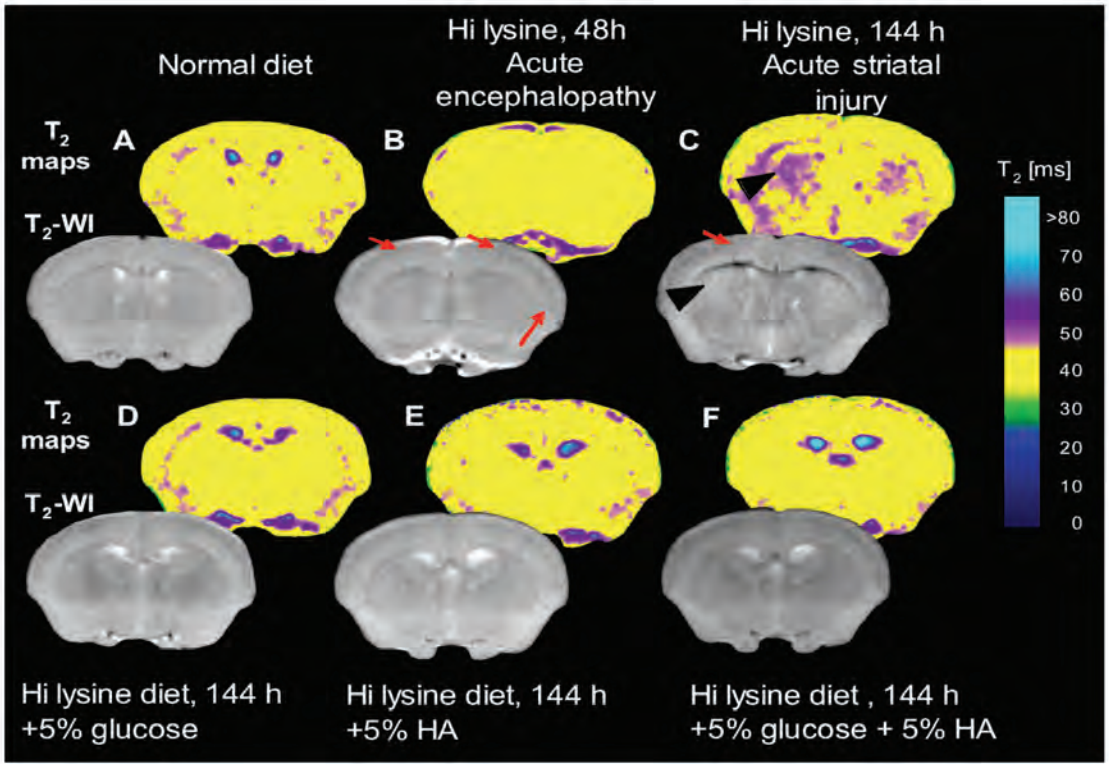


Figure 1. Representative T2-weighted images with corresponding T2 maps for Gcdh -/- mouse on normal diet (A) and corresponding treatments. The enlarged draining veins on T2-weighted image for Gcdh -/- on high lysine diet are present at 48 h, during acute encephalopathy (B arrows) and at 144 h (C). Evidence of striatal injury and accompanying increased T2-relaxation time become apparent at ~144 h after the onset of high lysine diet (C), black arrowhead. Animals that received glucose, homoarginine or both all have normal MRI (D, E, F).

76. Point-resolved and IZQ spectroscopy in MRS of the live mouse brain

Benoit Boulat, P.T. Narasimhan, Russell E. Jacobs

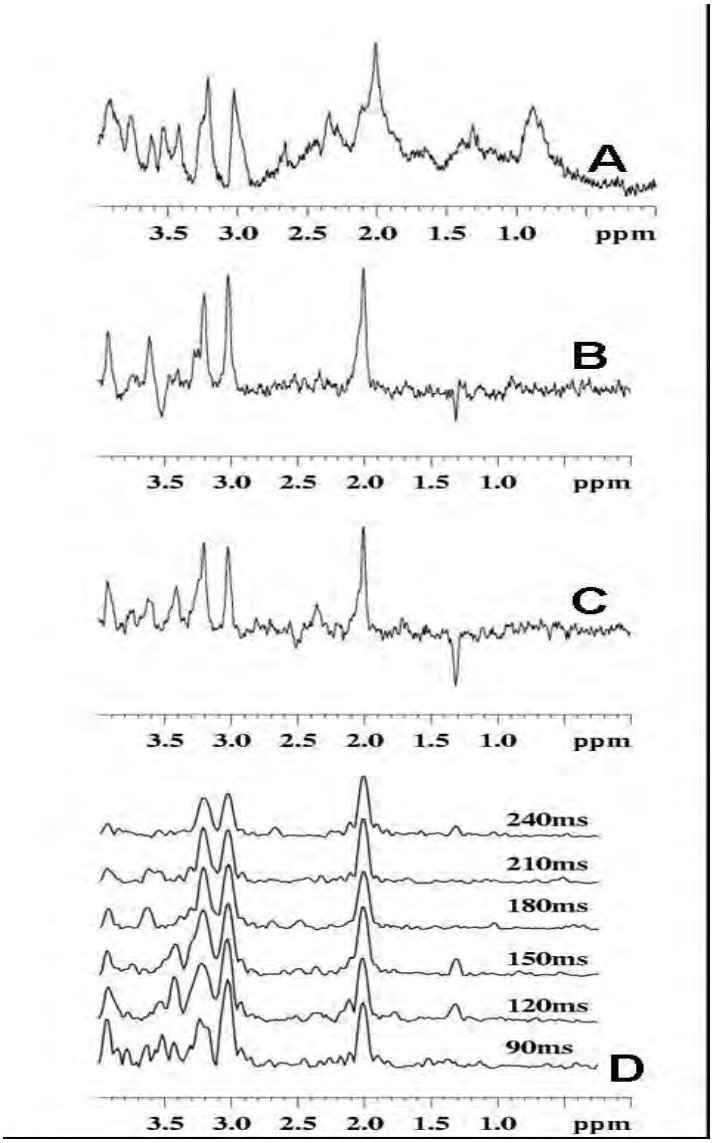
We have investigated the nature of the spectral information that can be gathered using two different Magnetic Resonance Spectroscopic (MRS) modalities in the live mouse brain at 11.7T. The first modality is the widely applied Point Resolved Spectroscopy (PRESS[1]) and the second one the Intermolecular Zero-Quantum (IZQ) spectroscopy. Representative PRESS spectra recorded in a 3mm^3 volume in the live mouse brain are displayed in panel A (echo time TE = 7.27ms), panel B (TE = 90ms) and panel C (TE = 120 ms). The quality of a PRESS spectrum in the live mouse brain deteriorates rapidly with volume size above 3 mm^3. A large number of narrow metabolite resonances seen in panel A are mixed with the broad macromolecular baseline, making quantification difficult. This issue is resolved with longer echo times but then the phase of scalar coupled resonances

complicates the results, as shown for the shifts around 3.5, 2.5 and 1.3ppm in panels B and C. Localized IZQ spectroscopy has proven impractical for volume size below 5mm^3. However, as displayed in panel D (absolute mode presentation) in a localized volume of 7mm^3 in the same mouse brain as in A, B and C, baseline issues are almost nonexistent. Moreover, by choosing different dipolar evolution times TD (noted on the right side above each IZQ spectrum), it is possible to focus on different metabolites which contain associated scalar coupled spin systems: myo-inositol (3.52-3.62ppm, TD=90ms-180ms), Taurine (3.25-3.42ppm, TD=120ms), glutamate (2.11-3.75ms, TD=120ms), glutamine (2.42-3.75ppm, TD=150ms), lactate (1.3ppm, TD = 120-150-240ms). Studying the levels of the above metabolites is of importance in mouse model of diseases such as Alzheimer, Parkinson and multiple sclerosis.

References

Bottomley, P.A. (1987) *Ann. N.Y. Acad. Sci.* **508**:333-348.

Vathyam, S., Lee, S. and Warren, W.S. (1996) *Science* **272**:92-96.



77. Live imaging of neuronal connections by MRI: Transport in a mouse model of Down Syndrome

Elaine L. Bearer[*]*, Xiaowei Zhang, Russell E. Jacobs*

Connections from hippocampus to septal nuclei have been implicated in memory loss and the cognitive impairment in Down syndrome (DS). We trace these connections in living mice by Mn^{2+}-enhanced 3D MRI and compare normal with a trisomic mouse model of DS, Ts65Dn. After injection of 4 nl of 200 mM Mn^{2+} into the right hippocampus, Mn^{2+}-enhanced circuitry was imaged at 0.5, 6, and 24 hr in each of 13 different mice by high resolution MRI to detect dynamic changes in signal over time. The pattern of Mn^{2+}-enhanced signal *in vivo* correlated with the histologic pattern in fixed brains of co-injected 3kD rhodamine-dextran-amine, a classic tracer. Statistical parametric mapping comparing intensity changes between different time points revealed that the dynamics of Mn^{2+} transport in this pathway were surprisingly more robust in DS mice than in littermate controls, with statistically significant intensity changes in DS appearing at earlier time points along expected pathways. This supports reciprocal alterations of transport in the hippocampal-forebrain circuit as being implicated in DS and argues against a general failure of transport. This is the first examination of *in vivo* transport dynamics in this pathway and the first report of elevated transport in DS.

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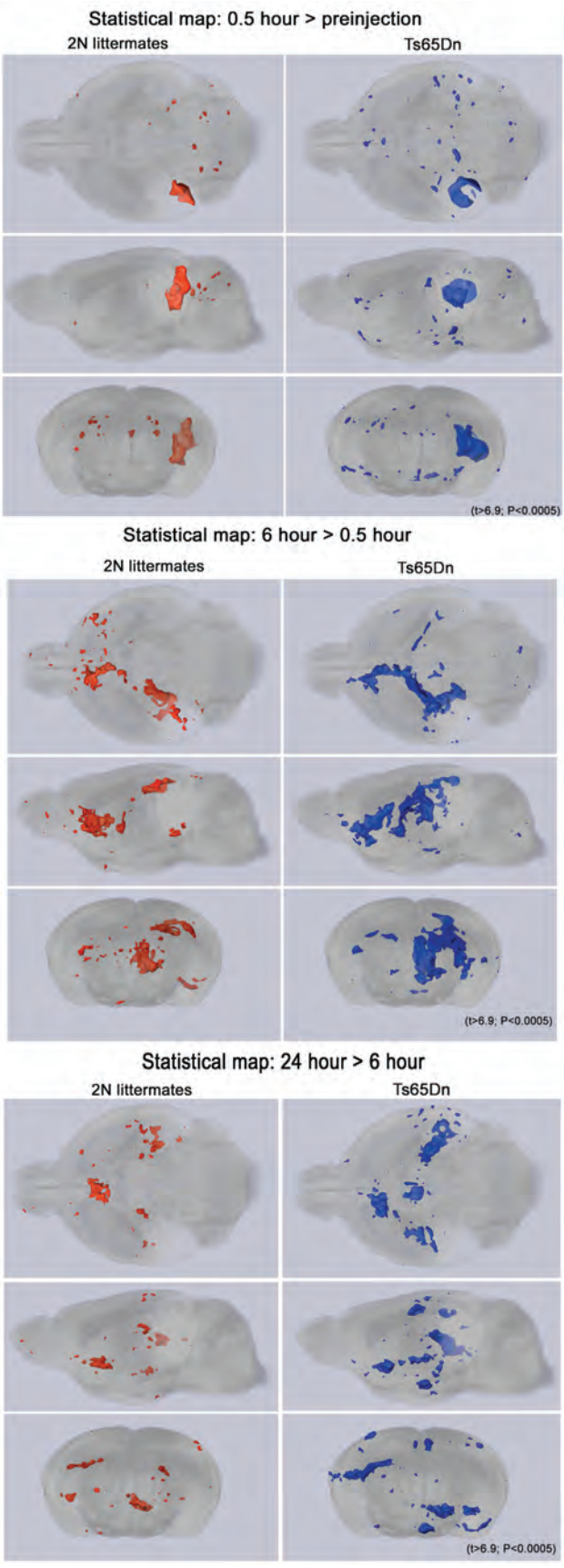
Increased Mn^{2+} transport in the hippocampal-forebrain pathway is apparent in statistical parametric mapping analysis of the MR data

Three views (top, side, end) of the same semitransparent volume projected into two dimensions are shown. Colored voxels (red for 2N and blue for DS) are those places where the intensity is significantly greater at the later time point ($p<0.0005$). Gray scale background is the 24-hour average image. (See next page)



78. Applications of terahertz imaging to medical diagnostics

Peter H. Siegel, Warren Grundfest[*]*, Scott E. Fraser*

This research program aims to apply terahertz (THz) source and sensor technology towards (1) establishing and cataloging properties and contrast mechanisms in biomaterials and tissues and (2) investigating potential disease diagnostic applications. THz waves occupy the wavelength region between 100 and 1000 microns, well beyond the traditional infrared. As a consequence they can penetrate more readily into many optically opaque materials including plastics, wood, clothing, some crystalline structures and, as is the emphasis for this program, a small number of biomaterials - lipids, bone, skin, hair, etc. Unlike infrared and optical signals, THz penetration in tissue is dominated by absorption not scattering, making the contrast mechanisms very different than those that are already being exploited in the optical regime. Water absorption is also extremely high (α =100-300 cm^{-1}) which has the disadvantage that very strong THz signal sources are required for significant tissue penetration, but the advantage that very subtle changes in fluid content are detectable (the basis for disease diagnostics). The first successful THz imaging systems have been based on picosecond pulsed-laser time domain spectroscopy techniques. These instruments offer unique spectral and time resolved information content but have limited penetration depth (tens of microns in tissue) and only modest signal-to-noise ratio (100-1000). This program has now demonstrated three types of THz imaging systems based on continuous wave and swept frequency heterodyne techniques (more generally used in space science and communications). One system operates at a fixed frequency of 2520 GHz (120 microns wavelength) and boasts a signal-to-noise of more than 10^{10}. A new frequency agile system, established two years ago, uses a millimeter wave vector network analyzer (MVNA) and can image between 100 and 1000 GHz (3 mm to 300 microns wavelength). Last year a swept frequency instrument, employing active illumination of the sample and real time transmission, reflection and/or scattering measurements over 15% bandwidth can perform both index of refraction or narrow band spectroscopy measurements on inserted samples between 550 and 650 GHz. This system uses all solid-state components, has hand held transmit and receive heads and portable electronics and power supplies. It is controlled by a lap top computer.

This year work is focusing on a new time domain system with instantaneous 2D imaging capability (rather than a single pixel scanned system) that can be used as a lead in to a real clinical application: THz detection of tumor margins and involvement in basal and squamous cell carcinoma. A pending NIH proposal will utilize this new equipment in a clinical study through USC Medical Center if it successfully passes the review process in 2007/8. The current program continues through September 2008.

*Dept. of Bioengineering, UCLA

This work is being funded by an NIH K25 training grant held by Dr. Siegel, who holds a joint appointment as a Technical Group Supervisor for Submillimeter Wave Advanced Technology (SWAT) at JPL and Faculty Associate in the Caltech Division of Engineering and Applied Sciences.

79. Flexible ribbon guide for *in vivo* and hand-held THz imaging

Peter H. Siegel, Cavour Yeh Scott E. Fraser*

Recent interest in terahertz frequency imaging for medical applications (wavelength range from 1 mm to 100 microns) has resulted in a flurry of new instrument proposals using both time domain and high-resolution frequency domain spectral techniques. However, to-date there exists no methodology for transporting terahertz signals from place-to-place with low loss, other than rigid-path-free space quasi-optical beam propagation. In order to take advantage of modalities common at optical wavelengths, including *in vivo* and portable hand-held sensor/receiver systems, the equivalent of signal-confining optical fiber links must be developed for the far infrared and terahertz bands. Commonly employed transparent materials in the visible (i.e., amorphous glasses or clear plastics) are all extremely lossy at longer wavelengths due to strong vibrational mode absorption. Dielectric substances with low absorption coefficient and high index do exist at terahertz frequencies, but they tend to be crystalline (quartz, silicon, germanium, GaAs) and therefore have poor mechanical properties when it comes to forming flexible guiding structures. Metallic waveguide (hollow or coaxial), although somewhat flexible, has untenable high resistive wall loss. A few plastics such as Teflon, polyethylene and polypropylene, have very low dispersion and absorption but have a low refractive index that makes it difficult to confine single mode terahertz energy as it propagates around bends or through joints.

Earlier work by our collaborators has shown that high index materials formed into ultra-thin ribbons can form very low loss guiding media at millimeter-wave frequencies (30-100 GHz). Extrapolating this concept into the terahertz bands, and taking advantage of modern fabrication techniques, we believe it is possible to use a combination of high-dielectric-constant crystalline materials in conjunction with low-loss, low index plastics to produce the equivalent of flexible terahertz optical fiber, i.e., "terahertz ribbon guide." Such a development would allow terahertz instruments to be freed from their fixed-beam-path table-top environment, enabling, for the first time at these wavelengths, both hand-held scanner and *in vivo* endoscopic applications. This task has been investigating both the design and fabrication of low-loss single-mode terahertz guiding structures. This year we have completed development of two new flexible THz waveguide structures and have fabricated both using a custom quartz extrusion process. The first structure is based on a simple hollow core waveguide cylinder but uses a thin quartz tube coated in metal as a flexible guide media. It has no advantages over traditional metallic waveguide except that it is flexible and inexpensive to fabricate. The second structure we call a cross bar encased ribbon guide. It uses the same extruded quartz material but propagates the ribbon guide mode in a sealed tube. Special single-mode launching structures have also been designed to match the RF fields into the two waveguides. RF measurements have not yet been performed but will take place this year.

* *California Advanced Studies*

This work is being funded by an NIH R21 grant to Dr. Siegel, who holds a joint appointment as a Technical Group Supervisor for Submillimeter Wave Advanced Technology (SWAT) at JPL and Faculty Associate in the Caltech Division of Engineering and Applied Sciences.

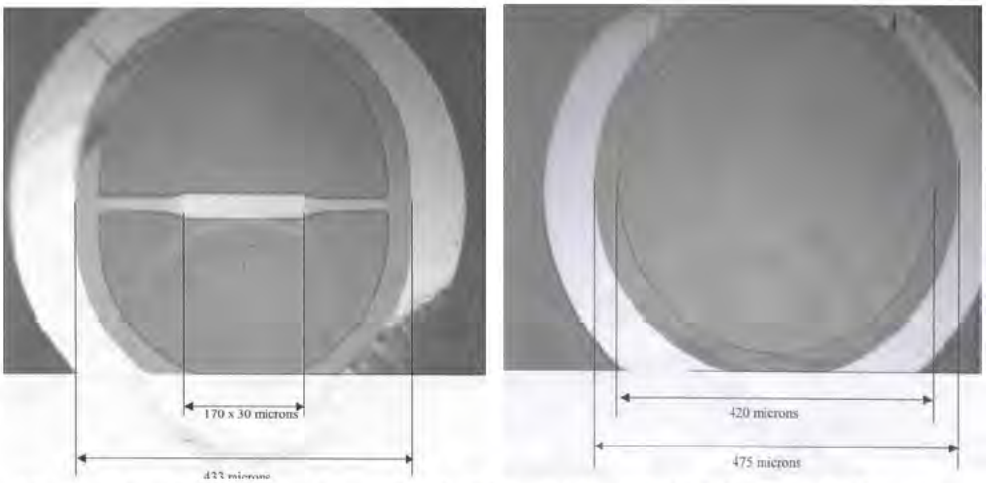


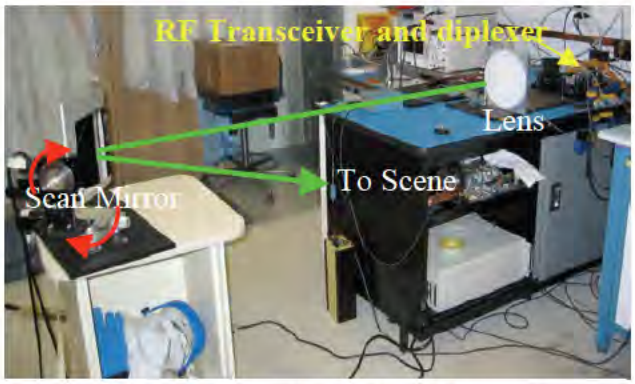
Photo (left) of new cross bar encased ribbon guide and (right) quartz hollow core waveguide for flexible propagation of terahertz waves.

80. Active submillimeter-wave imaging system

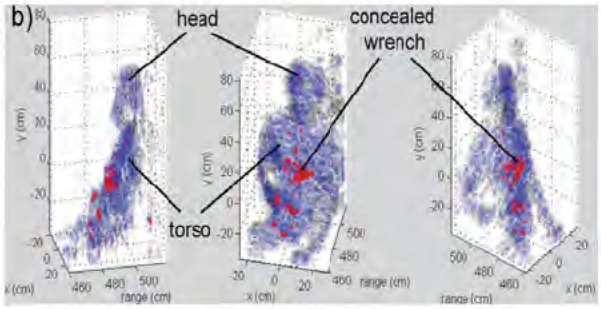
Peter H. Siegel, Scott E. Fraser - Members of the JPL Submillimeter Wave Advanced Technology Team

In 2006, we began a new program to leap frog our development of THz imaging components into a real world application. We began development of the first-of-its-kind THz imaging radar at a wavelength of 500 microns. The goal of the program is twofold: (1) to develop an active radar sounding instrument that can image in the Fresnel region (pre-far field) and allow range binning to sort out objects in the scene at different distances from the source and; (2) to apply this imaging technology to the assessment of security threats. The research is supported by the Department of the Navy. The components are based on technology that was developed for NASA space instruments targeted at performing passive radiometric measurements of chemical constituents in the Earth's atmosphere and around planets and small solar system bodies (comets and asteroids). The instrumentation greatly extends the capabilities of much lower frequency (millimeter wave) radar and applies these well-established techniques to the submillimeter wave bands for the first time. The application is doubly exciting because the radar techniques that are being developed can also be applied to future planetary sounders to perform absorption spectroscopy measurements that can identify biomolecules in the exosphere of several satellites, including Europa and Titan. As such it has already led to several additional pre-proposal efforts to internal funding sources at JPL and NASA.

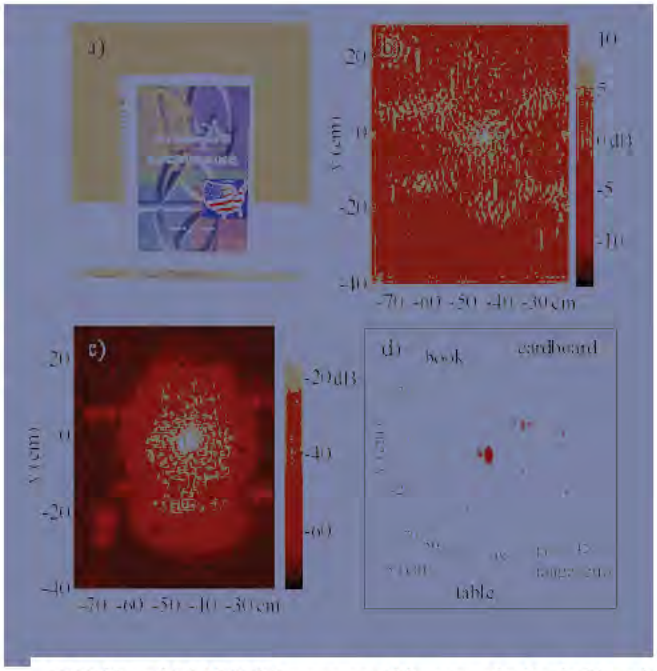
In the first nine months of the program, we successfully prototyped a 600 GHz (500 micron) frequency modulated continuous wave (FMCW) radar imager that can detect objects under clothing at 4m distance and allows complete 3D reconstruction and/or filtering of a scene. The final instrument will be targeted at the atmospheric transmission window centered at 670 GHz so that the range can be extended to 10-100 meters without suffering undue attenuation in the air. Some sample images are shown below.



RF System photo showing radar scanning and transceiver



3D profiles of a person at 4m range holding a concealed wrench under his shirt.



CW (b) and FMCW (c) range gated images of a book (a) and completely reconstructed scene (d). Figure shows how FMCW radar with the ability to perform range binning can bring out objects in the scene that are normally obscured by backscatter and clutter.

This work is being funded by a Navy grant from Physical Security Equipment Action Group (PSEAG) to Dr. Siegel, who holds a joint appointment as a Technical Group Supervisor for Submillimeter Wave Advanced Technology (SWAT) at JPL and Faculty Associate in the Caltech Division of Engineering and Applied Sciences.

81. Imaging the microorganization of synaptic receptors

Lawrence A. Wade, Daniel Lo, Scott E. Fraser, Henry A. Lester

Nicotinic acetylcholine receptors (nAChRs) are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR is a (pseudo) symmetric pentameric structure comprised of homologous subunits. Furthermore, the α subunits exist in at least ten different subtypes (α1 through α10) and the β subunits exist in at least four subtypes (β1 through β4) [1].

In this past year we've initiated an effort to combine several novel techniques to directly optically image the microorganization of AChRs expressed in *Xenopus* oocytes. By expressing different color XFPs in AChR α and β subunits, we expect to directly resolve and thereby determine the number of α and β subunits that comprise individual receptors. Furthermore, we hope to discretely identify the individual receptors over a field-of-view of at least one micron. This will enable us to simultaneously characterize the large-scale distribution of such receptors, to identify any local structures within an imaged membrane surface, and to assay the variation of receptor subtypes.

The techniques being combined in this experiment include a novel method for repeatedly locating imaging probes to specific substrate locations within a few 10's of nm [2], and an ~10 nm resolution, single-molecule sensitive near-field optical microscope [3,4]. A technique for attachment protein patterning on glass coverslips via Dip-Pen Nanolithography (DPN) [5,6] will be used to rigidly adhere the membranes to be image to a glass substrate. Receptor subunit imaging is enabled by the development of specific XFP-labeled AChR subunits [7][8].

By developing the ability to directly image each subunit of each AChR expressed in the membrane of *Xenopus* oocytes, we lay the groundwork for future studies of transmembrane proteins, receptors and membrane-embedded proteins in many different types of cells. In particular, we will substantially advance our ability to image the microorganization of nicotinic acetylcholine receptors at neuronal synapses.

Reference

[1] Tapper, A.R. *et. al*. (2004) *Science* **306**:1029-1032.

[2] Wade, L. (2006) Non-provisional US patent filed March 2006, Caltech Docket Number: CIT-4324.

[3] Gerton, J., Wade, L.A., Lessard, G., Ma, Z. and Quake, S. (2004) *Phys. Rev. Lett.* **93**:180801.

[4] Ma, Z., Gerton, J.M., Wade, L.A. and Quake, S.R. (2006) *Phys. Rev. Lett*. **97**:260801.

[5] Jung, H., Kulkarni, R. and Collier, C.P. (2003) *J. Am. Chem. Soc*. **125**(40):12096–12097.

[6] Jung, H., Dalal, C.K., Kuntz, S., Shah, R. and Collier, C.P. (2004) *Nano Lett.* **4**(11):2171-2177.

[7] Nashmi, R., Dickinson, M.E., McKinney, S., Jareb, M., Labarca, C., Fraser, S.E. and Lester, H.A. (2003) *J. Neurosci.* **23**:11554-11567.

[8] Fonck, C. *et. al.* (2005) *J. Neurosci.* **25**:11396-11411.

82. Field Resonance Enhanced Second Harmonic (FRESH) signaling biosensors

Periklis Pantazis, Ye Pu, John Hong[*], Demetri Psaltis[**], Scott E. Fraser

Signaling regulates embryonic development by providing positional information to cells so that they differentiate properly as they proliferate to build up the final shape. However, defects leading to abnormal activation of signal pathways often underlie most tumorigenic events. In recent years, much has been discovered about the molecular and biochemical characteristics of a variety of signal transduction pathways. In contrast, the cell biology of such signaling events is starting now to be studied. In particular, we are interested in the following questions: 1) when does signaling occur?; 2) where does it take place?; and 3) for how long? We aim to address these questions by developing new biosensors based on field resonance enhanced second harmonic (FRESH) for following *in vivo* signaling events with large sensitivity increase and high spatial and temporal resolution. We plan to use such biosensors to monitor signal transduction through visualization of conformational changes and protein-protein interaction of components of signaling pathways that will enable us to follow various signaling events in real time. Ultimately, the established biosensors will be used to investigate signaling during zebrafish development and in the manifestation of cancer.

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Publications

Canaria, C.A., So, J., Maloney, J.R., Yu, C.J., Smith, J.O., Roukes, M.L., Fraser, S.E. and Lansford, R. (2006) Formation and removal of alkylthiolate self-assembled monolayers on gold in aqueous solutions. *Lab on a Chip* **6**:289-295.

Damle, S., Hanser, B., Davidson, E.H. and Fraser, S.E. (2006) Confocal quantification of *cis*-regulatory reporter gene expression in living sea urchin. *Dev. Biol.* **299**:543-550.

Fingler, J., Williams, J., Yang, C.H. and Fraser, S.E. (2007) Imaging vasculature independent of direction of flow using spectral domain optical coherence tomography, Paper 6429-24, SPIE symposium: Coherence domain optical methods imaging of *Xenopus* gastrulation utilizing tissue-specific NMR image contrast. *Dev. Dynam.* **236**(2):494-501

Forouhar, A.S., Liebling, M., Hickerson, A., Nasiraei-Moghaddam, A., Tsai, H.J., Hove, J.R., Fraser, S.E., Dickinson, M.E. and Gharib, M. (2006) The embryonic vertebrate heart tube is a dynamic suction pump. *Science* **312**:751-753.

Koester, R.W. and Fraser, S.E. (2006) FGF signaling mediates regeneration of the differentiating cerebellum through repatterning of the anterior hindbrain and reinitiation of neuronal migration. *J. Neurosci.* **26**(27):7293-7304.

Kulkarni, R.P., Bak-Maier, M. and Fraser, S.E. (2007) Differences in protein mobility between pioneer versus follower growth cones. *Proc. Natl. Acad. Sci. USA* **104**(4):1207-1212.

Kulkarni, R.P., Castelino, K., Majumdar, A. and Fraser, S.E. (2006) Intracellular transport dynamics of endosomes containing DNA polyplexes along the microtubule network. *Biophys. J.* **90**(5):L42-L44.

Liebling, M., Forouhar, A.S., Wolleschensky, R., Zimmermann, B., Ankerhold, R., Fraser, S.E., Gharib, M. and Dickinson, M.E. (2006) Rapid three-dimensional imaging and analysis of the beating embryonic heart reveals functional changes during development. *Dev. Dynam.* **235**(11):2940-2948.

Liebling, M., Vermot, J., Forouhar, A.S., Gharib, M., Dickinson, M.E. and Fraser, S.E. Nonuniform temporal alignment of slice sequences for four-dimensional imaging of cyclically deforming embryonic structures. *Proceedings of the 2006 IEEE International Symposium on Biomedical Imaging: Macro to Nano (ISBI'06)*, Arlington, VA, USA, April 6-9, 2006.

Martin, M., Hiltner, T.D., Wood, J.C., Fraser, S.E., Jacobs, R.E., and Readhead, C. (2006) Myelin deficiencies visualized *in vivo*: Visually evoked potentials and T2-weighted magnetic resonance images of shiverer mutant and wild-type mice. *J. Neurosci. Res.* **84**(8):1716-1726.

Papan, C. and R.E. Jacobs (2007) Visualizing early *Xenopus* development with time lapse microscopic MRI. In: *International Textbook of In Vivo Imaging in the Vertebrate*, B. Tavitian, A. Leroy-Willig, and V. Ntziachristos, Eds., John Wiley & Sons, New York, New York. In press.

Papan, C., Boulat, B., Velan, S.S., Fraser, S.E. and Jacobs, R.E. (2007) 2D and 3D time-lapse imaging of *Xenopus* gastrulation utilizing tissue-specific NMR image contrast. *Dev. Dynam.* **236**(2):494-501.

Papan, C., Boulat, B., Velan, S.S., Fraser, S.E., and Jacobs, R.E. (2007) Formation of the dorsal marginal zone in *Xenopus laevis* analyzed by time lapse microscopic magnetic resonance imaging. *Dev. Biol.* **305**(1):161-171.

Papan, C., Tyszka, J.M. and Jacobs, R.E. (2007) Magnetic resonance imaging in developmental biology. In: *Molecular and Cellular MR Imaging*, Chapter 16, Modo, M.M.J.J. and Bulte, J.W.M. Eds, CRC Press, Boca Raton, FL. In press.

Tyszka, J., Readhead, C., Bearer, E., Pautler, R. and Jacobs, R. (2006) Statistical diffusion tensor histology reveals regional dysmyelination effects in the *Shiverer* mouse mutant. *Neuroimage* **29**(4):1058-1065.

Tyszka, J.M., Ruffins, S.W., Weichert, J.P., Paulus, M.J. and Fraser, S.E. (2006) Related methods for three-dimensional imaging. In: *Handbook of Biological Confocal Microscopy, third edition*, J. Pawley, editor, SpringerScience+Business Media, New York, NY.

Wang, J.B., Hamblet, N.S., Mark, S., Dickinson, M.E., Brinkman, B.C., Segil, N., Fraser, S.E., Chen, P., Wallingford, J.B., and Wynshaw-Boris, A. (2006) *Dishevelled* genes mediate a conserved mammalian PCP pathway to regulate convergent extension during neurulation. *Development* **133**(9):1767-1778.

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Summary: We are interested in multiple questions in basic and applied biology. For further information on Hay lab research consult our web page (http://www.its.caltech.edu/~haylab/). One goal of our work is directed towards understanding the genetic and molecular mechanisms that regulate cell death, proliferation, innate immunity and spermatogenesis. We use *Drosophila melanogaster* as a model system to identify genes that function to regulate these processes. Important cellular regulatory pathways are evolutionarily conserved; thus, molecules identified as regulators of these processes in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second goal of our work addresses three questions in population biology. 1) Can we bring about reproductive isolation (speciation) between populations of plants or animals that otherwise freely interbreed? Answers to this question have application to the growing number of situations in which plants and animals are engineered to show specific traits. In brief, we would like to be able to limit gene flow between engineered organisms and their wild counterparts. 2) Can we engineer the genetics of populations so that they drive themselves to local extinction? For example, invasive non-native plants and animals cause substantial economic losses. A number also cause substantial environmental damage, leading in many cases to extensive range reduction and/or extinction of unique, endemic species. Our goal is to develop genetic tricks that drive local extinction of invasive species. 3) Can we drive genes into wild populations so that all individuals express a trait of interest. We are particularly interested in developing transgenic insects that will prevent transmission of mosquito-borne diseases such as malaria and dengue fever. More than 500 million people are infected with the malaria parasite each year, resulting in more than 1 million deaths. Dengue, a mosquito-borne virus, infects more than 100 million people each year, resulting in more than 25,000 deaths. Effective vaccines for these diseases do not exist, and in the case of malaria, the causative agent, the parasite *Plasmodium falciparum* has acquired resistance to many drugs. Vector suppression through the release of sterile males, the use of insecticides, or modification of the environment provides an important tool for limiting mosquito-borne disease. However, each approach has limitations. Release of sterile males provides only transient population suppression, insecticides affect many non-target species and mosquitoes often evolve resistance to these compounds, and wholesale modification of the environment may not be feasible, or desirable, in many situations based on ecological concerns. Our goals are two-fold: to develop transgenic insects that lack the ability to transmit these pathogens; and to develop genetic tools for driving these genes into wild populations of insects, thereby blocking disease transmission.

83. *Drosophila* models of human neuro-degenerative diseases

Ming Guo (and the Guo lab), Bruce A. Hay

In collaboration with the Guo lab at UCLA we are studying *Drosophila* models of the two most common neurodegenerative diseases, Alzheimer's disease and Parkinson's disease.

References

Guo, M. *et al.* (2003) *Hum. Mol. Genet.* **12**:2669-2678.
Clark, I.E. *et al.* (2006) *Nature* **441**:1162-1166.

84. Gene activation screens for cell death regulators: MicroRNAs, small non-coding RNAs, define a new family of cell death regulator

Chun Hong Chen, Haixia Huang

We have carried out several screens for cell death regulators in the fly and have identified a number of new molecules. Among these are multiple microRNAs, small noncoding RNAs that function by inhibiting translation of target transcripts. We are interested in determining when and where these molecules regulate death, as well as the nature of their targets. We are also designing microRNAs that target known cell death regulators as a way of probing the function of these proteins in specific contexts.

85. Cell death, caspases and IAPs

Israel Muro, Jun Huh, Soon Ji Yoo, H. Arno J. Müller

In flies and vertebrates most, if not all, cells can undergo apoptosis in the absence of new gene expression, indicating that the components required to carry out apoptosis are present and ready for activation. The core of the cell death machine consists of members of a family of proteases known as caspases, which become activated in response to many different death signals. Active caspases then cleave a number of different cellular substrates that ultimately lead to cell death and corpse phagocytosis. Most if not all cells constitutively express caspase

zymogens (inactive precursors) sufficient to bring about apoptosis. Thus, the key to cell death and survival signaling revolves around controlling the levels of active caspases in the cell. Several basic strategies are used to regulate caspase activity, and the core proteins that drive caspase-dependent death are evolutionarily conserved. In *Drosophila* many cells experience chronic activation of the apical cell death caspase Dronc. If unrestrained, active Dronc cleaves and activates downstream effector caspases that bring about cell death. Cells survive because they express the IAP DIAP1, which suppresses Dronc activity, as well as that of caspases activated by Dronc. One major pathway through which caspase-dependent cell death in flies is induced is through the regulated expression of pro-apoptotic proteins that disrupt DIAP1-caspase interactions through several different mechanisms, each of which has the effect of unleashing a cascade of apoptosis-inducing caspase activity. We are interested in several questions. 1) What are the signals that lead to caspase activation in cells that would normally live? 2) How do IAPs regulate caspase activity and when and where does this regulation define points of control? 3) How is IAP activity regulated? 4) And finally, as discussed further below, how do caspases, IAPs and their regulators work to regulate non-apoptotic processes? We are using both genetic screens and biochemical approaches to identify the critical molecules.

86. Cell death in the fly eye

Jeffrey Copeland

Echinus is a *Drosophila* mutant that lacks normally occurring cell death in the eye. We cloned the *echinus* gene and found it encodes a member of an evolutionarily conserved family of proteins, the USPs, which remove ubiquitin from proteins. *Echinus* lacks interactions with known cell death regulators, suggesting that it acts at a novel point to promote death.

Reference

Copeland, J. M. *et al*. (2007) *BMC Dev.* **7**:82.

87. Caspases and their regulators in a non-apoptotic process, spermatid differentiation

Haixia Huang, Jun R. Huh, Jean Edens

We have found that multiple caspases, acting through distinct pathways, acting at distinct points in time and space, are required for spermatid individualization, a process in which spermatids (which develop in a common cytoplasm) become enclosed in individual plasma membranes and shed most of their cytoplasm*. Spermatid individualization is an evolutionarily conserved process, but little is known about how it is brought about. Several questions are of interest to us: 1) What are the upstream signals that drive caspase activation? 2) What are the nonapoptotic targets that facilitate differentiation? 3) How is cell death prevented in the face of high levels of caspase activity that would normally be associated with cell death? 4) Do caspases play similar roles in promoting spermatid differentiation in mammals?

Reference

*Huh, J. *et al*. (2004) *PLoS Biology* **1**:E15.

88. Cell death and the innate immune system

Jun Huh, Israel Muro, Chun Hong Chen, Ian Foe, Soon Ji Yoo, Jin Mo Park, Ming Guo

As discussed above, many IAP family proteins inhibit apoptosis. IAPs contain N-terminal BIR domains and a C-terminal RING ubiquitin ligase domain. *Drosophila* DIAP1 protects cells from apoptosis by inhibiting caspases. Apoptosis initiates when proteins such as Reaper and Hid bind a surface groove in DIAP1 BIR domains via an N-terminal IAP-binding motif (IBM). This evolutionarily conserved interaction disrupts IAP-caspase interactions, unleashing apoptosis-inducing caspase activity. DIAP2 overexpression also inhibits Rpr-and Hid-dependent apoptosis, but little is known about DIAP2's normal functions. We generated *diap2* null mutants, which are viable and show no defects in developmental or stress-induced apoptosis. Instead, DIAP2 is required for the innate immune response to gram-negative bacterial infection. DIAP2 promotes cytoplasmic cleavage and nuclear translocation of the NF-κB homolog Relish, and this requires the DIAP2 RING domain. Increasing the genetic dose of *diap2* results in an increased immune response, while expression of Rpr or Hid results in down-regulation of DIAP2 protein levels. Together these observations suggest that DIAP2 can regulate immune signaling in a dose-dependent manner, and that DIAP2 is regulated by IBM-containing proteins. Therefore, *diap2* may identify a point of convergence between apoptosis and immune signaling pathways.

Reference

Huh, J. *et al.* (2007) *J. Biol. Chem.* **282**:2056-2068.

89. Driving genes for disease refractoriness into wild pest insect populations

Chun Hong Chen, Haixia Huang, Catherine Ward, Jessica Su, Bruce Hay

An attractive approach to suppressing mosquito-borne diseases involves replacing the wild-insect population with modified counterparts unable to transmit disease. Mosquitoes with a diminished capacity to transmit *Plasmodium* have been identified in the wild and created in the laboratory, demonstrating that endogenous or engineered mosquito immunity can be harnessed to attack *Plasmodium*. However, a critical unanswered question is how to spread these effector genes throughout the areas inhabited by disease-transmitting insects. Epidemiological and modeling studies suggest that it will be necessary to rapidly replace a large percentage of the wild mosquito population with refractory insects in order to achieve significant levels of disease control. Because insect disease vectors are spread over wide areas and can migrate

significant distances, mass release of refractory insects associated with simple Mendelian transmission of effector-bearing chromosomes is unlikely to result in a high enough frequency of transgene-bearing individuals. Compounding this problem, enhancement of immune function in insects is often costly, requiring tradeoffs with other life history traits such as longevity and fecundity that decrease fitness. Therefore, it is likely that insects carrying effector transgenes will be less fit than their wild counterparts, resulting in a decrease in the fraction of individuals carrying genes for refractoriness over time. These observations argue that population replacement will require coupling of genes conferring disease refractoriness with a genetic mechanism for driving these genes through the wild population at greater than Mendelian frequencies.

Maternal-effect lethal selfish genetic elements have been described as genetic entities in the flour beetle *Tribolium casteneum*. The molecular nature of these elements (known as *Medea* elements) is unknown, but their genetic behavior makes them attractive candidates to mediate drive. This is because when present in a female, they must be inherited in the next generation in order for the offspring to survive.

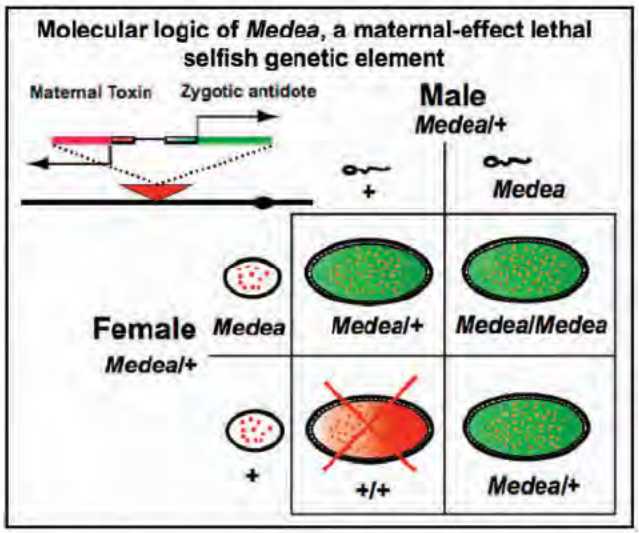

Figure 1. *Medea* is a "spiteful" selfish genetic element that enhances its transmission from generation to generation by causing the death of offspring that fail to inherit it. Mothers that carry a *Medea* element express a toxin (red dots) that is inherited by all oocytes (small ovals). Embryos (large ovals) that do not inherit *Medea* die because toxin activity (red background) is unimpeded (lower left square). Embryos that inherit *Medea* from the mother (upper left square), the father (lower right square) or both (upper right square), survive because expression of an antidote early during embryogenesis (green background) neutralizes toxin activity. *Medea* is comprised of two closely linked genes (upper left). One consists of a maternal germline-specific promoter that drives the expression of an RNA or protein that is toxic to the embryo. The second locus consists of a zygotic (early embryo) promoter that drives expression of an antidote.

This behavior is predicted to lead to rapid spread of the element within the population even if it carries an associated fitness cost because the chromosome that carries it gains a transmission advantage relative to counterparts that do not. Since the molecular biology of endogenous *Medea* elements is unknown, we created synthetic elements in *Drosophila* that can drive population replacement and that are resistant to recombination-mediated dissociation of drive and effector functions. The genetic and cell-biological principles utilized, which utilize microRNA-mediated silencing of a maternally-expressed gene essential for embryogenesis, coupled with early zygotic expression of a rescuing transgene, should be generally applicable to a number of other animal and plant species and have the potential to allow for iterative cycles of population replacement. With support from the Bill and Melinda Gates Foundation, we are expanding this work into the mosquito system.

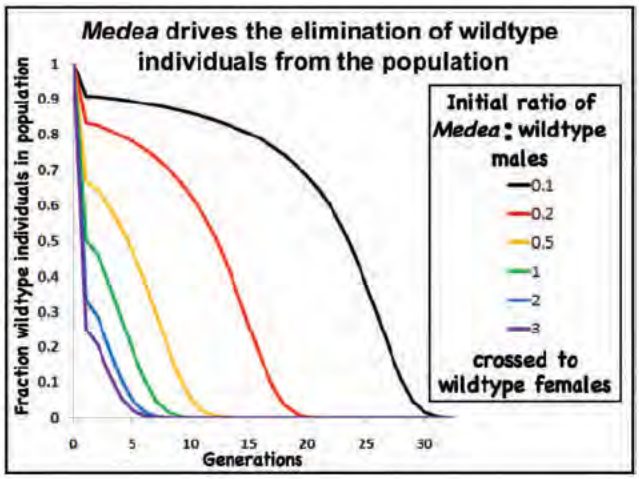

Figure 2. When *Medea*-bearing males are introduced into a population consisting of wildtype males and females, wildtype individuals are eliminated from the population. The greater the initial ratio of *Medea* to wildtype males, the more rapidly this elimination occurs.

Reference

Chen *et al.* (2007) *Science* **316**:597-600.

90. Sensing and killing dengue and yellow fever virus-infected cells in their insect host

Kelly J. Dusinberre

Dengue and yellow fever virus infect mosquitoes during a blood meal. The virus must enter and replicate inside mosquito midgut cells, disseminate throughout the body and ultimately infect the salivary gland (7-14 days later), in order to be transmitted to a new individual during a subsequent blood meal. Our goal is to develop transgenes that are phenotypically neutral when expressed in uninfected individuals, but that kill virus-infected cells and/or the mosquitoes themselves. The virus encodes several activities that are not present in uninfected host cells. These include a viral polyprotein protease, and

RNA-dependent RNA polymerase. We are developing molecules that sense these activities and cause the death of cells and insects in which they occur, thereby preventing disease transmission to humans.

Publications

Chen, C-H., Guo, M. and Hay, B.A. (2006) Identifying microRNA regulators of cell death in *Drosophila*. *Meths. Mol. Biol.* **342**:229-240.

Chen, C-H., Huang, H., Ward, C.M., Su, J.T., Schaeffer, L., Guo, M., Hay, B.A. (2007) A synthetic maternal-effect selfish genetic element drives population replacement in *Drosophila*. *Science* **316**:597-600.

Clark, I.E., Dodson, M.W., Jiang, C., Cao, J.H., Huh, J.R., Seol, J.H., Yoo, S.J., Hay, B.A. and Guo, M. (2006) *Drosophil*a *pink1* is required for mitochondrial function and interacts genetically with *parkin*. *Nature* **441**:1162-1166.

Copeland, J.M., Bosdet, I., Freeman, J.D., Guo, M., Gorski, S.M., Hay, B.A. (2007) *echinus*, required for interommatidial cell sorting and cell death in the *Drosophila* pupal retina encodes a protein with homology to ubiquitin-specific proteases. *BMC Dev. Biol*. **7**:82.

Hay, B.A. and Guo, M. (2006) Caspase-dependent cell death *in Drosophila*. *Ann. Rev. Cell & Dev. Biol.* **22**:623-650.

Huh, J.R., Foe, I., Muro, I., Chen, C-H., Seol , J.H., Yoo, S.J., Guo, M., Park, J.M. and Hay, B.A. (2007) The *Drosophila* inhibitor of apoptosis DIAP2 is required for the innate immune response to gram-negative bacterial infection, and can be negatively regulated by IAP-binding proteins of the Reaper/Hid/Grim family. *J. Biol. Chem*. **282**: 2056-2068.

Muro, I., Berry, D.L., Huh, J.R., Chen, C.H., Huang, H., Yoo, S.J., Guo, M., Baehrecke, E.H. and Hay, B.A. (2006) The *Drosophila* caspase *ice* is important for many apoptotic cell deaths and for spermatid individualization, a nonapoptotic process. *Development* **133**:3305-3315.

Yan, N., Huh, J.R., Schirf, V., Demeler, B., Hay, B.A. and Shi, Y. (2006) Structure and activation mechanism of the *Drosophila* initiator caspase Dronc. *J. Biol. Chem*. **281**:8667-8674.

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Summary: The Western world is experiencing a growing medical crisis. Epidemiologic and clinical reports reveal a dramatic increase in immune disorders: inflammatory bowel disease, asthma, type 1 diabetes, and multiple sclerosis. Emboldened by the 'hygiene hypothesis' proposed two decades ago, scientists have speculated that lifestyle changes (vaccination, sanitation, antibiotics) have predisposed developed societies to these disorders by reducing bacterial infections. However, the hypothesis remains without explanation as our exposure to most bacteria does not result in disease. Mammals are colonized for life with 100 trillion indigenous bacteria, creating a diverse ecosystem whose contributions to human health remain poorly understood. In recent years, there has been a revolution in biology toward understanding how (and more importantly, why) mammals harbor multitudes of symbiotic bacteria. We have recently demonstrated for the first time that intestinal bacteria direct universal development of the immune system; thus fundamental aspects of mammalian health are inextricably dependent on microbial symbiosis. Furthermore, it is now clear that all of the diseases in question astonishingly involve a common immunologic defect found in the absence of symbiotic bacteria. As we have co-evolved with our microbial partners for eons, have strategies used against infectious agents reduced our exposure to health-promoting bacteria, ultimately leading to increased disease? We propose that the human genome does not encode all functions required for health, and we depend on crucial interactions with products of our microbiome (collective genomes of our gut bacterial species). Through genomics, microbiology, immunology and animal models, we wish to define the molecular processes employed by symbiotic bacteria that mediate protection from disease. Advances in the past year have now made it possible to mine this untapped reservoir for beneficial microbial molecules. Ultimately, understanding the immune mechanisms of these *symbiosis factors* may lead to natural therapeutics for human diseases based on entirely novel biological principles.

91. Dynamic surface variation by symbiotic bacteria is required for host colonization

Sung-Eun Lee, Janet Chow, Richard H. Jones, Julie Huang

Bacterial surfaces represent functional organelles decorated with molecules that mediate critical interactions between the microbes and their milieu. These environments may be on or within another organism; not uncommonly, that organism is a mammal. Capsular polysaccharides are abundant external structures of bacteria, and the capsules of many prokaryotic pathogens have been found to be important virulence factors during mammalian infection. Unlike pathogens, commensal bacteria establish a life-long co-habitation with their mammalian hosts. However, the molecular mechanisms employed to establish this beneficial relationship remain almost entirely undescribed. The unique identification of multiple surface polysaccharides in the important human symbiont *Bacteroides fragilis* raised the critical question of how these molecules contribute to commensalism. Herein, we report that mutation of the master regulator of *B. fragilis* polysaccharide expression results in a global reduction of capsule. Surprisingly, attempts to completely eliminate expression of capsule are not tolerated and result in abrogation of bacterial growth. Subsequently, the organism acquires a spontaneous mutation that restores growth and production of at least one capsular polysaccharide. We identify an alternative pathway by which *B. fragilis* is capable of re-establishing capsule production. Most importantly, mutants expressing single, defined surface polysaccharides remain defective for intestinal colonization of animals compared to bacteria that express a complete polysaccharide repertoire, a process mediated by specific interactions between bacteria and intestinal mucus. The extensive surface diversity and multiple layers of regulation suggest a profound evolutionary requirement for capsular polysaccharide during host-bacterial symbiosis.

92. Host-bacterial mutualism by a microbial symbiosis factor prevents inflammatory disease

June L. Round, Juscilene Menezes, Sara McBride

Colonization of humans with multitudes of commensal species creates an ecosystem harboring members of five of the six kingdoms of life. Bacteria in particular dominate this ecologic niche; the gastrointestinal tract is resident to an astounding $>10^{14}$ microorganisms with a diversity of approximately 2,000 species. This consortium of gut bacteria represents an integral factor in mammalian biology. Germ-free animals, born and raised under sterile conditions, exhibit profound defects in the development of intestinal tissues. Many reports have shown that both gastrointestinal and systemic immune responses are deficient in the absence of commensal microoroganisms. Surprisingly however, the gut is stably colonized by both beneficial and potentially pathogenic microorganisms; the reasons for this phenomenon remain unclear. Moreover, imbalances in the composition of the

bacterial microflora, known as dysbiosis, are thought to be a major factor in human disorders such as inflammatory bowel disease (IBD). We report herein that the ubiquitous human symbiont, *Bacteroides fragilis*, protects animals from experimental colitis induced by the pathogenic commensal, *Helicobacter hepaticus*. Most importantly, this beneficial activity requires a single bacterial molecule (Polysaccharide A or PSA). Animals harboring *B. fragilis* not expressing PSA develop disease and produce pro-inflammatory cytokines in colonic tissues similar to *H. hepaticus* colonization alone. Purified PSA administered to animals protects from experimental colitis and wasting disease by inducing anti-inflammatory responses, both *in vivo* and *in vitro*, and activation of interleukin 10-producing $CD4^+$ T cells. These results reveal the first molecule of intestinal commensal bacteria that mediates the critical balance between health and disease. As incidence of IBD have dramatically increased over the last several decades, harnessing the immunomodulatory capacity of *symbiosis factors* such as PSA may ultimately provide novel therapeutics for human inflammatory disorders.

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Summary: "Nor you, nor I, nor anyone knows, how oats, peas, beans, and barley grow." *Traditional children's song*

This is a problem – although every person on Earth depends entirely on growth of plants for food, and plants also provide fiber, building materials, feedstocks for the chemical industry, and, increasingly, sources of energy, we know amazingly little about how they grow. In part this is due to neglect – only 2% of the Federal support for basic research in the life sciences goes to work on plants. But it is also due to the difficulty of the problem – there are many things we need to learn about plant gene function, genomes, and cell-cell communication, and the effects of both the internal and external environments on them, before we can begin to understand plant growth.

In our laboratory we don't use crop plants to study plant growth, we concentrate instead on *Arabidopsis thaliana*, a small member of the mustard family that is easy to grow indoors in large numbers, and whose genome is fully sequenced and annotated. It doesn't grow differently from other plants as far as we know, but it is much easier to study. The part of growth on which we concentrate is growth from the tips of shoots, which is where all above ground growth comes from. At the tip of each shoot is a collection of stem cells, both in the biological sense and the literal sense, called the shoot apical meristem. This collection of a few hundred cells serves two general functions. First, it provides the cells that serve as founder cells for the stem, including its vasculature, and for leaves and flowers. The founder cells of a flower are the floral primordium; in *Arabidopsis* the time from primordial establishment to a mature flower is less than two weeks. Secondly, cell divisions in the shoot apical meristem replace the cells that have departed from the meristem to make stem, leaves, and flowers. These cell divisions are tightly controlled, so that the meristem remains about the same size through the life of each shoot.

To better understand shoot apical meristems and the early development of flowers and leaves we have developed a series of new methods and approaches. Our major advance in information gathering is the use of a laser scanning confocal microscope and associated software to do live imaging of the cells and domains of gene expression in the shoot apical meristem and in developing leaves and flowers. Along with this we have developed methods for activating or inactivating particular genes while we are watching (Reddy *et al.*, 2007, in the Publication List reviews these and related methods), and also for using a dye laser to ablate individual cells (or groups of cells) in the shoot apical meristem during our live observations. We are also developing computational methods to deal with two problems that the flood of live imaging data presents. One is image processing – using automated methods to recognize and count cells, quantitate gene expression and protein levels, and to represent the three-dimensional array of cells in the meristem over time. The other is computational modeling. With an abundance of information, and the demonstration of many levels of feedback in the interactions of cells with each other, the movement of hormones in the meristem, and in the activation and repression of specific genes, our hypotheses must now be explicit and quantitative (that is, mathematical models that run in computers), so that we can get specific predictions of meristematic behavior at the level of single genes and single cells. This allows us in turn, to stringently test the hypotheses by comparing the output of a model in the computer with the output of a cognate experiment in living plant tissue. The output of experiments then serves as data for tuning the parameters of the models (and changing the models themselves) to better fit experimental results. By this iterative process, observation in plants, model development in computers, model testing *in silico*, model testing *in planta*, then back to model development - we are developing a detailed understanding of plant growth. Our computational work is a collaboration with a large international group of coworkers, as the author list of the abstracts should make clear.

In the past year we have applied our live-imaging methods to the initial formation of shoot apical meristems in tissue culture, to the maintenance of the meristem, to the formation and growth of floral and leaf primordia, and to floral organ primordium (sepal) growth. Models for meristematic cell maintenance and for the positioning of new floral primordia have been made, and have been shown to be successful in predicting experimental outcomes. We also continue, as always, to find new mutations that reveal the genes and processes at work in plant growth, so that the activity of these new genes can be added to our growing models. The eventual goal of all of our work is a full computer representation of the activity of each gene, and each cell, in a living plant - a representation we term, The Computable Plant.

93. Pattern formation during *de novo* assembly of the *Arabidopsis* shoot meristem

Sean P. Gordon, Elliot M. Meyerowitz

Most organisms have a capacity to regenerate lost features, however, few have the ability to regenerate an entire new body plan from adult tissue. Induction of new shoot meristems from cultured root explants is a widely used but poorly understood plant regeneration system in which the apical portions of new plants are regenerated from adult somatic tissue through the *de novo* formation of new shoot meristems. We have characterized early patterning events during regeneration of the *Arabidopsis* shoot meristem using fluorescent reporters of known gene and protein activities required for embryonic and post-embryonic shoot meristem development and maintenance. We find that a small number of progenitor cells initiate development of new shoot meristems through stereotypical stages of reporter expression and activity of *CUP-SHAPED COTYLEDON 2 (CUC2)*, *WUSCHEL (WUS)*, *PIN-FORMED1* (*PIN1*), *SHOOT-MERISTEMLESS* (*STM*), *FILAMENTOUS (FIL)*, *REVOLUTA (REV)*, *ARABIDOPSIS THALIANA MERISTEM L1 LAYER (ATML1)*, and *CLAVATA3 (CLV3)*. We also demonstrate a functional requirement for *WUS* activity in early regeneration as the mutant is defective in early steps of the regeneration process. We propose that shoot meristem regeneration is an easily accessible system for the investigation of molecular mechanisms of *de novo* patterning and complex regeneration in the well-studied model organism, *Arabidopsis*.

94. Cytokinin induction of the *WUSCHEL (WUS)* gene in *Arabidopsis*

Sean P. Gordon, Kaoru Sugimoto, Elliot M. Meyerowitz

Tissue culture experiments have shown that expression of the *WUSCHEL (WUS)* gene is induced by culture on medium with high levels of the plant hormone cytokinin. We propose that cytokinin treatment is sufficient to induce *WUS* expression, even in tissues where it is not normally expressed. We intend to test this hypothesis by inducing ectopic *WUS* expression in root explants cultured *in vitro*. We will further investigate the necessity of cytokinin for ectopic *WUS* expression by monitoring the induction of *WUS* in explants mutant for cytokinin receptors or downstream components of the cytokinin-signaling pathway. Furthermore, we are investigating a requirement of light for the putative induction of *WUS* from root tissues as cytokinin-induced genes have been shown to stabilize active forms of light receptors, required for the perception of environmental light cues.

95. Identification of putative factor X, downstream of the *WUSCHEL (WUS)* gene in *Arabidopsis*

Sean P. Gordon, Elliot M. Meyerowitz

The *WUSCHEL (WUS)* gene is required for stem cell maintenance at the tip of the shoot meristem in *Arabidopsis* and therefore endows the shoot meristem with the ability to continuously generate new above ground organs during post-embryonic development. However, *WUS* is expressed in the inner cells of the shoot meristem and therefore must act in a non-cell-autonomous manner to maintain stem cells at the apex of the shoot meristem. One possible hypothesis is that *WUS* expression induces the expression of an unknown factor X that is secreted by inner cells and travels to the shoot apex where it acts to maintain shoot stem cell identity. We propose to identify the putative factor X using a shoot regeneration assay. We will induce high levels of *WUS* expression using transgenic plant cells over expressing *WUS* in liquid culture. We will purify the supernatant of this liquid culture and use it for *in vitro* shoot regeneration assay to test for the presence of a factor capable of rescuing shoot regeneration in samples mutant for the *WUS* gene. If such a factor is found we will further investigate its molecular nature (i.e., protein or small molecule) by its sensitivity to protease degradation, and chemical fractionation and mass spectrometry may also be used.

Publications

Gordon, S.P., Heisler, M.G., Reddy, G.V., Ohno, C., Das, P. and Meyerowitz, E.M. (2007) Pattern formation during *de novo* assembly of the *Arabidopsis* shoot meristem. *Development.* In press.

Reddy, G.V., Gordon, S.P. and Meyerowitz, E.M. (2007) Unravelling developmental dynamics: transient intervention and live imaging in plants. *Nature Rev. Mol. Cell Biol.* **8**:491-501.

96. Relative hormone levels determine spatial organization of shoot formation in *Arabidopsis*

Sean P. Gordon, Vijay S. Chickarmane, Elliot M. Meyerowitz

Recent experiments in our laboratory are consistent with a model in which non-homogenous distributions of auxin and cytokinin within tissue explants induces the formation of shoots. Our model assumes that the inhomogeneity in auxin/cytokinin is 'read' by a core genetic network, whose genes then express proteins which subsequently feedback on hormone synthesis to enhance auxin/cytokinin gradients. Thus, the combined genetic/signaling network is an "intensifier" a network that first perceives a relative difference in hormonal levels and then further acts to accentuate the difference. Two genes which display behavior predicted in our model for genes active in an auxin/cytokinin feedback loop are *CUP-SHAPED COTYLEDON 2* (*CUC2*) and *WUSCHEL* (*WUS*). *CUC2* and *WUS* appear to be expressed in opposing gradients that are predictive of progenitor/non-progenitor cell fates and are induced after auxin and

cytokinin treatment, respectively. Confocal images of tissue where these proteins were tagged, seem to indicate that CUC2 and WUS are mutually antagonistic, and whose levels can be toggled by relative levels of auxin/cytokinin. We also assume that CUC2 and WUS positively feed back on the synthesis of auxin and cytokinin, respectively. We have developed a computational model based upon partial differential equations that exhibit stable patterns of CUC2 and WUS from initial auxin/cytokinin inhomogeneities. A generalized result from such Turing-like pattern formation problems is the diffusion rates of participating species are quite different. We hypothesize that this is achieved in our system through the expression of PIN efflux proteins, which would tend to concentrate auxin in a region where it is already expressed and hence, slow down its effective diffusion rate. In a combined experimental/computational approach we seek to verify several links in our model, and to establish whether relative hormone levels can indeed provide the cue for cell differentiation and in particular shoot regeneration.

97. How do veins form in leaves? A model based upon recruitment and differentiation using auxin transport by PIN efflux agents

Vijay S. Chickarmane, Henrik Johnsson, Marcus Heisler, Eric Mjolsness, Elliot M. Meyerowitz

A fundamental question is how venation patterns arise in a leaf. Recent experiments suggest that even before the cells become procambial (vein cells), patterns of auxin and membrane-localized PIN proteins are formed. Cells with higher auxin content ultimately become vein cells, thereby reinforcing the venation patterns. Earlier models have used flux-based models to describe vein formation in leaves. Although they are successful in describing the key features of the patterns, they are not based upon any known molecular mechanisms, and hence are not amenable to genetic experiments. We have been developing a multicellular model, which assumes a reaction network in each cell and which includes active transport of auxin. The model hypothesizes that "receptor" molecules in each "vein cell" (operationally defined as a cell with PIN localized on its membrane), gets localized towards the neighboring cell with maximum auxin. This recruited cell localizes its PIN proteins to face the receptor molecules. Hence each "vein" cell recruits a single cell from a field of cells, which subsequently itself becomes a "vein cell." In our model, we describe the evolution of PIN, auxin, receptor molecules, and influx agent AUX1 levels by a set of differential equations. Numerical simulations in 2-D show PIN localization patterns that are consistent with the occurrence of loops of auxin flux. The latter are observed experimentally. We are currently testing various hypotheses: autoregulatory sources of auxin; locating auxin sources are at the tip and on the sides of the leaf; and having the receptor network interrogate auxin concentration in neighboring cells. This last hypothesis has very strong positive feedback, which under certain circumstances, would imply that once vein formation has occurred, it may not be able to change in response to further local application of auxin.

98. Interactions between MSL2, MSL3, and the plastid division machinery

Elizabeth S. Haswell, Elliot M. Meyerowitz

Mechanosensitive (MS) ion channels provide a mechanism for the perception of mechanical stimuli such as sound, touch, and osmotic pressure. The bacterial MS ion channel MscS opens in response to increased membrane tension and serves to protect against cellular lysis during osmotic downshock. MscS-like proteins are found widely in bacterial and archaeal species, and have also been identified in fission yeast and plants. We characterized two MscS-Like (MSL) proteins from *Arabidopsis thaliana*, MSL2 and MSL3 (Haswell *et al.*, 2006). MSL3 can rescue the osmotic shock sensitivity of a bacterial mutant lacking MS ion channel activity, suggesting that it functions as a mechanosensitive ion channel. *Arabidopsis* plants harboring insertional mutations in both *MSL3* and *MSL2* show abnormalities in the size and shape of plastids, plant-specific endosymbiotic organelles responsible for photosynthesis, gravity perception, and numerous metabolic reactions. MSL2-GFP and MSL3-GFP are localized to discrete foci on the plastid envelope, and co-localize with the plastid division protein AtMinE. To follow up on these results we have investigated the genetic and cell biological interaction between the plastid division protein MinD and the MS ion channels MSL2 and MSL3. A mutation in MinD, *arc11*, is synthetic lethal with *msl2*-1 and *msl3*-1, while other mutations affecting plastid size do not show any genetic interaction with these alleles. In addition, MinD-YFP is mis-localized in the epidermal plastids of *msl2-1; msl3-1* mutant plants. MinD-YFP signal is found in the stroma of mutant plastids, rather than at the envelope, as in wild-type plastids. Further analysis of the interactions are underway.

Reference

Haswell, E.S. and Meyerowitz, E.M. (2006) *Curr. Biol.* **16**:1-11.

99. Functional analysis of the *msl2*-1 and *msl3*-1 mutant alleles

Elizabeth S. Haswell, Susana Nava, Elliot M. Meyerowitz

The ten MscS-Like (MSL) proteins of *Arabidopsis* show sequence similarity to the bacterial mechanosensitive ion channel MscS. MscS functions as an osmotic safety valve, allowing the non-selective release of ions upon extreme hypoosmotic shock. To determine the function of two of these MSL proteins, MSL2 and MSL3, insertional alleles were isolated from pools provided by the University of Wisconsin (Haswell *et al.*, 2006). msl2-1; msl3-1 double mutant plants have variegated leaves and enlarged chloroplasts. However, in both *msl2-1* and *msl3-1* alleles, the insertion is located in the last exon of the gene, downstream of the transmembrane domains and the conserved MscS domain.

As the levels of *MSL2* and *MSL3* mRNA are normal in *msl2-1* and *msl3-1* mutants, we are taking several approaches to investigate the nature of the defect in these mutants. Genomic and cDNA sequencing suggest that the *msl2-1* and *msl3-1* mutant alleles produce truncated proteins lacking the extreme C-termini of MSL2 and MSL3. Truncated MSL3 (MSLt) functions normally in an *E. coli* osmotic shock assay, suggesting that ion channel activity per se is not disrupted. Rather, intraplastidic localization is disrupted in the truncated proteins, as MSL3t-GFP and MSL2t-GFP are dispersed around the periphery of the plastid envelope instead of clustered at the poles as with the full-length versions. Furthermore, over-expression of MSL3t or MSL2t in a wild-type background phenocopies the *msl2-1; msl3-1* double mutant. Comparison of the *msl2-1* and *msl3-1* alleles with a recently identified loss-of-function allele of MSL2 and with MSL3 RNAi lines is underway. In the future, we plan to identify proteins that interact with the C-termini of MSL2 and MSL3, and to elucidate the causal relationship between plastid morphology, MSL2/3 localization, and the C-terminus of MSL2/3.

Reference

Haswell, E.S. and Meyerowitz, E.M. (2006) *Curr. Biol.* **16**:1-11.

100. The role of MSL9 and MSL10 in the osmotic shock response of the *Arabidopsis* root

Elizabeth S. Haswell, Rémi Peyronnet[*], Jean-Marie Frachisse[*], Hélène Barbier-Brygoo[*], Elliot M. Meyerowitz

MscS is a bacterial mechanosensitive (MS) ion channel that functions as a safety valve, allowing ions to exit the cell and preventing plasmolysis in response to extreme hypoosmotic shock. MSL9 and MSL10 are two members of the MscS-Like (MSL) protein family in *Arabidopsis*. MSL9-GFP and MSL10-GFP fusion proteins are localized to the plasma membrane and to endosomes when expressed under their endogenous promoters. Treatments known to induce hyper-osmotic shock signaling, such as salt, osmotica, or the plant hormone abscisic acid, repress the expression of MSL9 and MSL10. RT-PCR analysis demonstrated that MSL10 is widely expressed throughout the plant, while MSL9 expression is primarily expressed in the tip of the root. GUS reporter lines verify these data and further refine the expression patterns, showing complimentary expression patterns for the two genes. The MSL10p::GUS reporter gene is expressed strongly in the vasculature of the root and shoot, while MSL9p::GUS activity is found in the columella cells of the root cap and the cortex and endodermal cell layers of the root elongation zone. Low levels of GUS activity are also found at the base of young leaves, but appear to be excluded from the vasculature in MSL9p::GUS plants. We also have direct evidence that MSL9 and MSL10 are mechanosensitive ion channels that respond to changes in membrane tension. A stretch-activated ion channel activity has been characterized in root protoplasts using the whole cell method. This activity is increased by negative pressure in the patch pipette (suction) and is inhibited by treatment with 1mM gadolinium ion, an inhibitor of MS ion channel activities. This activity is absent in *msl9-1; msl10-1* double mutant plants and can be restored by transient expression of MSL9 or MSL10. Taken together, these data are consistent with the hypothesis that MSL9 and MSL10 play a role in the osmotic shock response of the *Arabidopsis* plant, and to our knowledge is the first time a protein directly required for a MS ion channel activity in plants has been characterized.

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Reference

Haswell, E.S. (2007) *Curr. Topics Membranes* **58**:329-359.

101. A genetic screen for enhancers of *msl2-1*

Elizabeth S. Haswell, Elliot M. Meyerowitz

The *Arabidopsis MSL2* and *MSL3* genes encode putative mechanosensitive (MS) ion channels related to the bacterial MS ion channel MscS. No phenotype has yet been detected in single *msl2-1* or *msl3-1* mutant plants, but *msl2-1* and *msl3-1* double mutants have variegated leaves, enlarged chloroplasts, and enlarged, spherical epidermal plastids (Haswell *et al.*, 2006). Variegation, or the presence of different colored tissues in the leaf, has been observed in a number of mutants and is frequently attributed to defects in plastid development. It has been proposed that a plastid-to-nucleus signal prevents proper leaf development when plastid development is disrupted. The *msl2-1; msl3-1* mutant is unusual in that it is variegated but chloroplasts are normally developed, through greatly enlarged. Other mutants with enlarged plastids, such as the *arc (accumulation and replication of chloroplasts)* mutants also have enlarged plastids, but none of them exhibit variegation. To begin to understand the nature of the variegation pattern observed in the *msl2-1; msl3-1* double mutant, we have initiated a genetic screen for variegated mutants in the *msl2-1* single mutant background. 20,000s seed were treated with 0.4% of EMS for 8 hrs, washed thoroughly, and planted. M2 seed was collected in pools of 40 and 160 seed from each of 10 pool planted. Seed from 56 mutant plants with visible variegation was collected. We are now verifying these phenotypes in the M3, performing backcrosses, complementation tests, and generating mapping populations. Mutants that phenocopy the *msl2-1* and *msl3-1* mutant in that they have enlarged chloroplasts are likely contain new alleles of MSL3, or genes that control MSL2 or MSL3 function. Mutants that are variegated but do not have enlarged chloroplasts may contain lesions in genes downstream of MSL2 in the putative plastid-to-nucleus signal transduction pathway. We are interested in both classes of mutants, and both classes are present in our mutant collection.

Reference
Haswell, E.S. and Meyerowitz, E.M. (2006). *Curr. Biol.* **16**:1-11.

102. Auxin modelling in the shoot apical meristem

Marcus Heisler, Henrik Jönsson[*]

One notable feature of our auxin transport model (Jönsson *et al*., 2006) is that when the model is modified to include the experimental result that PIN1 transcription is auxin regulated, the resulting auxin distribution patterns are not stable. Secondly, simulations of auxin distribution in the meristem indicate that if PIN1 is predominantly expressed in the L1, auxin levels should be lower there than in cell layers below, assuming synthesis and degradation is ubiquitous. However, RT PCR and microarray results have shown that the auxin influx carrier AUX1 is also auxin regulated and expressed in the meristem. Expression analysis of AUX1 mRNA using *in situ* hybridization as well as visualization of a functional YFP-AUX1 reporter protein in fact indicate that AUX1 is predominantly expressed in the L1 layer of the meristem and upregulated during the early stages of primordium specification. We therefore extended our model to incorporate AUX1 and to have both AUX1 and PIN1 regulated by auxin. Simulation results show that the incorporation of auxin-regulated AUX1 restores stability to the model when PIN1 is also auxin-regulated. We conclude that AUX1 probably functions redundantly with closely related AUX1-like proteins both to maintain auxin levels in the meristem epidermis and stabilize auxin distribution patterns generated by PIN1-mediated auxin gradient models.

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Reference
Jönsson *et al.* (2006) *Proc. Natl. Acad. Sci. USA* **103**:1633-1638.

103. Investigation of local signalling for determination of PIN1 polarity

Marcus G. Heisler, Olivier Hamant[*]*, Jan Traas*[*]

A central tenet of our auxin transport model is that cells signal to their neighbors their auxin content such that cells can position PIN1 according to the relative auxin concentration in neighboring cells. To start to test this hypothesis we investigated the effects of local cell ablation using a micro-pulsed laser beam. We have found that PIN1 responds to local ablation events by polarizing away from wounded cells. This response does not appear to be position specific and is not emulated by other membrane-localized proteins such as AUX1 or PINOID. Interpreting this in terms of our model, if we assume dead cells are incapable of signaling positive auxin concentrations to their neighbors, the result that PIN1 polarizes away from ablated cells should be emulated by our model and simulations so far bear this out. Surprisingly, however, similar responses are seen in *monopteros* (mp) mutant plants. MP encodes an Auxin Response Transcription Factor (ARF) thought to be required for auxin transcriptional responses in the meristem. The observation that PIN1 reorientations occur despite the lack of MP function suggests that either these reorientations do not require auxin-induced transcription or that other MP-like genes function somewhat redundantly with MP to mediate the response.

Elements of the cytoskeleton are also known to respond to wounding. Wounding experiments on injured pea roots indicated that cortical arrays of microtubules (MTBs), originally aligned with their helical axis along the apical/basal axis of the root, reorient their axis to being perpendicular to the wound edge (Hush *et al*., 1990). This occurs within 5 hrs, which is a similar time frame to the reorientation observed for PIN1. To investigate the similarity in response between PIN1 and MTBs in response to wounding we conducted laser ablations of cells while imaging a GFP-MAP4 marker line in the *Arabidopsis* meristem. MTB orientation was observed to respond to wounding by orienting their helical axes radially from wounded cells within 5 hrs confirming that MTB behavior displays striking similarities to PIN1 behavior in response to wounding. Furthermore, we noticed that MTB orientations throughout the meristem epidermis appear to be very similar to the orientation of PIN1 polarity suggesting that either these cellular components influence one another's behavior or that they may share upstream regulatory factors. We are presently examining PIN1 and MTBs together to gauge the extent of their co-alignment in detail and are investigating possible causal interactions.

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Reference
Hush *et al.* (1990) *J. Cell Sci.* **96**:47-61.

104. Establishment of adaxial/abaxial patterning of lateral organs in *Arabidopsis*

Marcus G. Heisler, Carolyn Ohno

A fundamental patterning process in plant and animal development is the positioning of lateral organs and their partitioning into distinct cell types. In plants the dorsoventral boundary of organs such as leaves defines an axis of symmetry through the center of the organ separating the upper (dorsal or adaxial) and lower (ventral or abaxial) organ tissues. Although the mechanism responsible for defining the position of this boundary is unknown, these and other observations have suggested that dorsoventral boundary establishment may be related to the process that defines organ position. By imaging GFP reporter genes we find that lateral organ primordia are positioned on a pre-existing boundary of abaxial gene activity that includes *KANADI1* (*KAN1*), a gene known to promote ventral cell-type specification, and miRNAs antagonistic to *REVOLUTA*, within the peripheral regions of the shoot apical meristem (SAM). The expansion of adaxial gene activity from the meristem center to abut this boundary occurs subsequently and is likely promoted by auxin. These data demonstrate that plant organ cell-type

patterning along the adaxial/abaxial axis is directly inherited from a meristem pre-pattern that defines the outer boundary of the organogenic peripheral zone.

105. Characterization of the callus-forming cells in plant *in vitro* regeneration

Kaoru Sugimoto, Sean Gordon

Plant cells have been recognized as totipotent because they are competent to become the full array of plant tissues, unlike in the case of animal regeneration. In the commonly used *in vitro* plant regeneration assay, first, cells are induced to proliferate as an unorganized cell mass (callus) from a small piece of tissue explant on callus-inducing medium. Subsequent culture of the callus on shoot- or root-inducing medium causes the cells to be specified and differentiate into shoot or root tissues, respectively. Because cells are thought to acquire competency and proliferate as they form callus, callus formation might be a key event in plant regeneration. However, little is known about what kind of cells contribute to the formation of callus and what might be their differentiation status when they divide to form the callus. We plan to characterize the cells that form callus by investigating the gene expression patterns of these cells, using the technology of live imaging and whole-genome microarrays. To determine the origin of callus-forming cells, we plan to follow the expression of tissue-specific markers and cell division markers. There are three possibilities for the origin of callus-forming cells: fully specialized cells de-differentiate; pre-existing cells with higher competence proliferate; or both. So far, we have observed the expression pattern of root tissue markers in root explants and the results support the third possibility. More detailed observations of these genes and other tissue markers are now underway. In addition, by microarray analysis, we will investigate if the callus-forming cells are similar to meristematic or embryonic tissues, which give rise to new organs in normal plant development, and how cells are different from the differentiated cells of the organs from which the callus is derived. We will compare callus formation from the cells of several kinds of organs, such as root, leaf and petal, to determine what elements of callus formation are general and what changes are tissue specific. Already we have found the conditions required for inducing callus from these organs.

106. Dissection of the flower initiation pathway using genetic and genomic approaches

Wuxing Li

Understanding of the regulation of fate determination and patterning in plants and animals requires insight into their genetic regulatory networks. The shoot apical meristem in *Arabidopsis* provides an excellent system to study processes in cell division, cell differentiation, and cell fate determination. Understanding of how floral meristem fate is determined is of significant agricultural importance, as well as answering fundamental questions in biology. I propose to systematically characterize the process of the floral transition utilizing both genetic and genomic approaches. Transcription networks will be examined during the floral transition using expression profiling and dynamic change patterns of global transcription will be analyzed. Genes that either promote or repress the vegetative-to-floral transition will be incorporated to build mathematical models that explain the floral transition process. Chromatin status during this process will also be examined and the involvement of genes, either the regulators of chromatin status, or the genes whose expression is regulated at the chromatin level, will be determined.

107. Genetic studies of the expression pattern of the floral regulatory gene *LEAFY*

Wuxing Li

Recent studies have identified the *Arabidopsis* gene *LEAFY* as a master regulator in specifying floral meristem identity. I have started a detailed characterization of this gene: (1) factors that control temporal and spatial pattern of *LFY* expression; (2) upstream components of *LFY* function; and, (3) genes that interact genetically or biochemically with *LFY*. The expression pattern of *LFY* has been observed in different mutant background and an auxin function-related gene was found to potentially regulate *LFY* spatial expression. Through a mutagenesis study of a weak *lfy-5* allele, I have obtained several putative novel genetic modifiers, and further characterization of these modifiers should provide new insight into the floral initiation pathway. It is also expected that yeast two-hybrid experiments will elucidate new components in the pathway of LFY function. This research will advance our understanding in fundamental questions such as cell fate determination.

108. Cell fate decision by CLAVATAs in shoot apical meristem

Xiang Qu, Zachary L. Nimchuk, G. Venugopala Reddy[*]*, Elliot M. Meyerowitz*

All the aerial parts of the plant body that develop after germination depend on the activity of the shoot apical meristem (SAM). Meristematic tissues consist of small cells with dense cytoplasm and relatively large nuclei that act like stem cells in animals. It is crucial for plants to achieve a balance between cell division and cell differentiation in these cells to maintain a functional meristem.

Arabidopsis serves as a model system for discovery of the mechanism by which functional SAMs are maintained in plants. We now have a general description of the molecular processes that lead to the homeostasis between meristematic cell division and differentiation during plant growth and development as a result of a negative feedback regulation in a two-way signaling system: a hypothetical *CLAVATA* (*CLV1*; a transmembrane receptor kinase, *CLV2*; a receptor-related gene; and *CLV3*, the putative CLV1 ligand) signal transduction that restricts meristematic cell number; and the homeodomain *WUSCHEL* (*WUS*, a homeodomain transcription factor)-mediated pathway which promotes

cell division within the SAM. However, little is known of real actions of genes like *CLVs* during meristematic reorganization and maintenance at the molecular level. To acquire a better understanding of how CLVs act in concert during SAM growth and development, we have generated constructs that allow us to visualize and trace the changes in expression of CLVs at both RNA and protein levels. We have been using a combination of methodologies (biochemical, genetic, and cell biological) to dissect and trace the primary and secondary (and beyond) effects of the mutated or misexpressed CLVs. A mutant-based approach has also been under taken to reveal the relationships among CLV1, CLV2 and CLV3 during SAM maintenance.

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109. Dynamic analysis of the GATA-like transcription factor HANABA TARANU during *Arabidopsis* development

Yuanxiang Zhao[*], Elliot M. Meyerowitz

HAN (*HANABA TARANU*) encodes a GATA-like transcription factor and is essential for normal floral development in *Arabidopsis*. All four identified *han* mutants (*han-1*, *han-2*, *han-3*, and *han-4*) display dramatic floral phenotypes, with fused sepals and reduced organ number in all four floral whorls. The expression pattern of *HAN* in the shoot apical meristem (SAM) and floral meristem is distinctive, with strong expression at the boundaries between the meristem and its newly initiated organ primordia, and at the boundaries between different floral whorls. Although *han* mutations have minor effects upon vegetative SAMs, *han; clv* double mutants display highly fasciated (overgrown) SAMs. Along with the observation that the *WUS* expression pattern is altered in *han* mutants, our data suggest that HAN is also involved in SAM development. To monitor the action of HAN during floral and SAM development, we have developed a hormone-inducible system which allows us to activate HAN by treatment with dexamethasone (DEX). Following the activation of *HAN*, we can track changes in expression pattern of selected floral and SAM identity genes (*LFY*, *WUS*, for example) by use of live imaging. In addition, to elucidate the genetic interactions between *HAN* and other known floral and SAM identity genes, we have generated various double and higher-order mutants by crosspollination.

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110. Identification of genetic partners of HANABA TARANU by second-site mutagenesis

Elliot M. Meyerowitz

The phenotype of *han* mutants is pleiotropic. Mutations in *HAN* affect floral meristem size, floral organ number and identity, and floral organ separation, indicating HAN plays multiple roles in floral development. In addition, the *HAN* expression pattern in the shoot apical meristem (SAM), and observations that mutations in *HAN* enhance *clv* mutant phenotypes and cause aberrant *WUS* expression in *han* mutants, imply that HAN is also involved in SAM maintenance. To understand the molecular mechanism by which HAN regulates flower and SAM development, in collaboration with Dr. Wolfgang Lukowitz at Cold Spring Harbor Labs, we have conducted a second-site mutant screen for suppressors from the EMS-mutagenized *han-2* plants. About 20,000 mutagenized plants from 40 pools were screened, among which, 64 repressors and 25 enhancers were identified. One of the *han-2* enhancers (gene *X*) displays a severe phenotype. The floral organs of the double mutant consist of only carpels. Beside the floral phenotype, the mutant plants display a miniature growth habit, loss of apical dominance and greatly reduced numbers of cauline leaves. The single mutant of *X* displays an apetala-like phenotype. Further analysis and mapping these mutants is currently under way.

111. Live imaging of the formation of giant cells in the *Arabidopsis* sepal

Adrienne Roeder, Vijay Chickarmane

One of the fundamental mysteries of developmental biology is how neighboring cells are specified to have different fates and how they differentiate to achieve those fates. For example, how does one cell know to become greatly enlarged while its neighbor remains small and how is the difference in sizes generated? We are addressing this question in the *Arabidopsis* sepal epidermis where cells range in size from the smallest cells, which reach only 1% the length of the sepal, to the extremely elongated giant cells, which can stretch half the length of the sepal. We observe that the cell size is correlated with the nuclear DNA content. Larger cells have more DNA suggesting that they have undergone endoreduplication, a specialized cell cycle in which the cell undergoes additional rounds of DNA replication without the usual intervening cell division that occurs during the normal mitotic cell cycle. Although all of the cells expand, the small cells remain petite because their volumes are halved at each cell cycle while the endoreduplicating cells become enlarged as they continue to grow through each cell cycle. We propose that the range of cell sizes in the sepal epidermis is generated by temporal differences in the initiation of endocycles. The earlier a cell enters endoreduplication, the more endocycles it can complete and the larger it becomes. Currently, we are testing this possibility through computational modeling and by observing the growth and cell division pattern of living sepals. A single sepal on a living plant was imaged every 12 hrs for 7.5 days on a confocal microscope while it developed. In the time period observed, many small cells divided, but the giant cells never divided supporting our model that giant cells become enlarged by starting endoreduplicating earlier than their smaller neighbors.

112. The ACR4 receptor kinase controls giant cell development

Adrienne Roeder

To identify genes involved in the development of the extremely elongated giant cells in the *Arabidopsis* sepal epidermis we conducted a screen for mutants defective in giant cell development. We isolated three classes of mutants: mutants without giant cells; a mutant with too many large cells; and mutants with smaller giant cells. We have focused on two allelic mutants in which fully elongated giant cells are absent in the sepals. We have observed, however, that smaller giant cells appear to develop in the sepals of these mutants. The nuclei in the smaller giant cells of the mutant are not as large as wild-type giant-cell nuclei suggesting that they have undergone fewer endocycles correlating with their smaller size. On the molecular level, a giant cell-specific marker is expressed in the smaller giant cells present in the mutant. A second marker expressed specifically in the non-giant pavement cells of wild-type sepals is similarly excluded from the smaller giant cells of the mutant. These results suggest that giant-cell fate is specified, at least partially, in the mutant, but that the full expansion of the giant cells fails to occur. Through positional cloning we have found that these mutations are in the *Arabidopsis CRINKLY4* gene, which encodes a receptor kinase involved in epidermal and ovule development (Tanaka *et al.*, 2002; Gifford *et al*., 2003; Watanabe *et al*., 2004; Cao *et al.*, 2005; Gifford *et al*., 2005), suggesting that a signaling pathway is involved in giant cell development.

References

Cao, X., Li, K., Suh, S.-G., Guo, T. and Becraft, P.W. (2005) *Planta* **220**:645-657.

Gifford, M.L., Dean, S. and Ingram, G.C. (2003) *Development* **130**:4249-4258.

Gifford, M.L., Robertson, F.C., Soares, D.C. and Ingram, G.C. (2005) *Plant Cell* **17**:1154-1166.

Tanaka, H., Watanabe, M., Watanabe, D., Tanaka, T., Machida, C. and Machida, Y. (2002) *Plant Cell Physiol.* **43**:419-428.

Watanabe, M., Tanaka, H., Watanabe, D., Machida, C. and Machida, Y. (2004) *Plant J.* **39**:289-308.

113. Genome-wide study of mRNA decay during floral development

Yuling Jiao, Jose Luis Riechmann

The abundance of mRNAs is controlled at the transcriptional level and also at the level of RNA degradation. Although the transcriptional controls of mRNAs have been extensively studied, relatively little is known about regulation of gene-specific degradation. It is generally believed that decay of mRNA is primarily through 5' decapping and followed by 5' to 3' degradation by exonuclease, whereas 3' to 5' degradation functions as a complementary pathway. In addition, plant miRNA targets are usually cleaved in the 5' region and the 3' cleavage products are subject to 5' to 3' degradation.

Many genes and miRNAs are expressed in floral tissues in *Arabidopsis*. We are developing a novel method to comprehensively detect 5' decapped mRNAs, which are targets of miRNAs and decapping enzymes. This approach takes advantage of the fact that these decapped mRNAs lack protective 5'-caps and carry a 5' phosphate group. An adaptor ligation-based protocol is used to enrich decapped and 5' phosphorylated mRNAs. Identities of such RNA are found using an oligonucleotide microarray. We will use a genetically engineered floral induction system for synchronized floral development to study the decay of mRNAs during early floral development.

114. Whorl-specific transcriptome during floral development

Yuling Jiao

The *Arabidopsis* flower is composed of four whorls, which develop distinct types of floral organs. Although key homeotic transcription factors controlling specification of floral organ type have been genetically identified, the immediate downstream transcriptional networks initiated by the homeotic genes are largely unclear. This is primarily due to the complexity of developing flowers – different transcription factor combinations are active in each whorl, and therefore different downstream events occur in each whorl. We are developing a system to study the whorl-specific transcriptome during early floral development. Homeotic genes *AP1*, *AP3* and *AG* have well-defined whorl-specific expression patterns during flower development, under the control of their promoters. An epitope-tagged ribosomal protein is expressed under these promoters in specific whorls. Full-length polysomal mRNAs are expected to be co-purified through immunopurification of polyribosomal complexes and can be used for global gene expression analysis (Zanetti *et al*., 2005). A previously developed floral induction system has been recruited to enable collection of synchronized flower buds during early development stages for genome-scale profiling (Wellmer *et al.* (2006).

References

Wellmer *et al.* (2006) *PLOS Genetics* **2**:1012-1024.

Zanetti *et al*. (2005) *Plant Physiol.* **138**:624-635.

115. Global mapping of homeotic transcription factor-binding targets

Yuling Jiao

It is well accepted that homeotic transcription factors determine organ identities during floral development. It would be interesting to identify the direct-binding targets of these transcription factors to fully understand the transcriptional network immediately downstream of these key regulators. In addition to these homeotic genes, transcription factors SEPALLATA1, 2, 3, and 4 (SEP1, SEP2, SEP3 and SEP4) are redundantly required for flower development as they form tetramers with whorl-specific homeotic transcription factors AP1,

AP3/PI or AG to confer proper organ identifies. Genetic studies indicate that SEP3 is the major SEP protein. To identify direct-binding targets of these transcription factor tetramers, we expressed epitope-tagged SEP3 in plants to enable immunoprecipitation of SEP3 together with its binding genomic DNA. The identities of these SEP3-binding targets will be combined with gene expression profiles to understand the binding effects on transcription. To further reveal differences among the whorl-specific tetramers, AP1, AP3 and AG promoters will be utilized to generate whorl specifically expressed SEP3. These lines are being developed and will be used for further dissection of the SEP3 targets when SEP3 is combined with different homeotic transcription factors.

The assay for the immunoprecipitated genomic DNA will include hybridization to promoter or tiling path microarrays, and also direct sequencing using the Illumina highly parallel sequencing technology now in operation in the Biology Division's Millard and Muriel Jacobs Genetics and Genomics Laboratory. Future experiments will include use of the immunoprecipitation assays in a genetic background where different floral homeotic transcription factors will be inactivated by mutation, which could reveal how the binding activity of SEP3 is affected by its being complexed with other proteins. The binding targets will be further compared with expression profiles to distinguish direct interactions and secondary downstream effects.

116. MODIFIER OF B FUNCTION gene negatively regulates expression of petal and stamen organ identity genes in *Arabidopsis* flower development

Carolyn Ohno, Marcus Heisler

The developmental expression of homeotic patterning genes in both plants and animals is under strict regulation both spatially and temporally to ensure that proper organs arise in appropriate positions. In plants, the mechanisms that give rise to stable whorl-specific expression patterns of the B function petal and stamen floral organ identity genes have not yet been revealed. The putative transcription factor MODIFIER OF B FUNCTION (MOB) was identified as a repressor of the B function gene *PISTILLATA* (*PI*) and as a genetic enhancer of the *curly leaf (clf)* mutant in a screen for modifiers of floral homeotic transcription in *Arabidopsis*. While the two B function proteins PI and APETALA3 (AP3) are heterodimer partners, only *PI* is ectopically expressed in both flowers and vegetative tissues such as leaves and stem in the *mob* mutant. Fluorescently-tagged MOB protein accumulation in wild type, *ufo* mutant or constitutively expressed *UFO* transgenic lines is nuclear-localized and ubiquitous, indicating that MOB protein stability is not regulated post-transcriptionally by UFO. Uniform expression is consistent with *MOB* mRNA expression and suggests that MOB and UFO act in parallel pathways in which the repression of *PI* by *MOB* does not require UFO activity. MOB does not require UFO or LFY for activation of B function genes and may function in a pathway independent of UFO and LFY. Our data reveal that *MOB* alone appears to be sufficient to negatively regulate the initiation and maintenance of *PI* transcription, while the synergistic interaction of *mob* and *clf* double mutants suggests MOB and the chromatin histone methyltransferase CLF function together to regulate initiation and maintenance of both *PI* and *AP3* expression.

117. JAGGED transcriptional targets for leaf growth

Carolyn Ohno

To understand growth and morphogenesis of plant lateral organs, we have continued characterization of JAGGED (JAG), a putative zinc-finger transcription factor that is expressed in leaf and floral organ primordia. Loss of *JAG* function results in the development of narrow jagged leaves and floral organs due to defects in both cell divisions and cell expansion. In contrast, misexpression of *JAG* in the floral meristem promotes the development of leaf-like tissue in place of flowers, suggesting that *JAG* is sufficient to specify leaf growth. To identify downstream transcriptional target genes of *JAG* that may be involved in specifying leaf cell fate or cell proliferation, transcriptional profiling using whole genome microarrays has revealed genes whose expression is altered in transgenic *jag* mutant seedlings after inducible expression of *JAG*. One candidate JAG transcriptional target upregulated after induction of *JAG* is a giberellin (GA) 2-oxidase gene involved in GA metabolism. The expression of a GA 2-oxidase reporter gene overlaps with *JAG* expression in developing wild-type sepals, and appears to be reduced in *jag* mutants, consistent with the possibility that the GA 2-oxidase may be positively regulated by *JAG*. To assess whether *JAG* activity is mediated by GA 2-oxidase activity, experiments that misexpress the GA 2-oxidase in order to rescue the *jag* mutant phenotype are underway. Genetic characterization of multiple mutants within the family of *Arabidopsis* GA 2-oxidase genes is also being assessed to address possible redundancy among these genes. Microarray analyses have also indicated that a KNOX gene may be negatively regulated by *JAG* induction. It will be interesting to determine if both GA regulation and KNOX activity converge as a direct result of the JAG transcriptional response.

118. A role for histone modification in the regulation of sepal organ number

Zachary L. Nimchuk, Elliot M. Meyerowitz

Proliferation of cells in plants is restricted to specific regions called meristems. These regions give rise to all the above ground organs of plants (shoot and floral meristems) and below ground organs (root meristems). In floral meristems, cells of the meristem are consumed during formation of floral organs. Floral organs arise in regular spaced whorls of distinct organ number and identity. We have isolated a mutant which we have named *SNOWBALL* in the Ws-0 background. *SNO* mutants display various defects indicative of altered meristem function. Most prominently, *SNO* mutants display alterations in floral organ number with increases in sepal

numbers. *SNO* acts independently of, or additively with *CLV1*, *STM* and other meristem regulatory mutants. Genetic evidence suggests that *SNO* acts redundantly with *PID*, a regulator of auxin transport. *SNO* was mapped and cloned and encodes HDA19, a histone deacetylase. Based on sequencing, the *sno* mutant is likely to encode a protein null of *HDA19*. *hda19* null mutants have been identified in other ecotype backgrounds but increases in sepal number have not been described. Preliminary analysis indicates that Col-0 background *hda19* null plants have wild-type sepal numbers. *SNO* has been crossed to Col-0 *hda19* mutants to assess if a Ws-0-specific modifier is responsible for sepal number increases in *sno*. In addition we are exploring the role of auxin in *SNO* sepal specification by examining markers for auxin and auxin-dependent specification in WT and *sno*.

119. Cell biological, genetic and biochemical approaches to studying the CLAVATA1 receptor kinase

Zachary L. Nimchuk, Xiang Qu, Elliot M. Meyerowitz

Plants grow via the constant proliferation of cells from specific regions termed meristems. The shoot meristem gives rise to all above ground tissues. In the shoot meristem the rate of cell proliferation within the meristem is balanced with the allocation of cells into lateral organs. Thus, the net exit of cells from the meristem is balanced with new cell production in order to maintain the meristem proper. Several classes of mutants exist in *Arabidopsis* that affect in this balance. Mutations in *CLV1* lead to a hyper-accumulation of stem cells in both shoot and floral meristems resulting in stem fasciation, club-shaped siliques and extra floral organs. *CLV1* encodes a receptor-like kinase (RLK) with extracellular leucine-rich repeats and a cytoplasmic serine-threonine (ser-thr) signaling domain that may interact with the putative peptide ligand CLV3. Although the *CLV* class-of-mutants have been around for many years, little is known about CLV1 regulation or signaling. We are taking several complementary approaches to study CLV1 function. Using a range of tagged CLV1 alleles we define roles for different domains in CLV1 function at the genetic and cellular level. Our analysis indicates that the extracellular domain and kinase activity are required for CLV1 function. In addition, we have found a class of intra- and extragenic mutations that appear to regulate the activation of the CLV1 pathway. We are in the process of defining the role of these genes in CLV1 activation.

120. HD-ZIP transcription factor GLABRA2 and its putative lipid/sterol binding domain

Kathrin Schrick, B.P. Venkata[1], Sara A. Klemin[1], B.P. Srinivas[2], Martin Hülskamp[2]

GLABRA2 (GL2) is an end-pathway homeodomain leucine zipper (HD-ZIP) transcription factor in a complex regulatory circuit controlling patterning and cell-type differentiation of the epidermis of *Arabidopsis*. As a representative member of the plant-specific class IV HD-ZIP transcription factor family, GL2 is postulated to be regulated post-translationally via a putative lipid/sterol-binding domain termed START (for Steroidogenic acute regulatory protein (StAR)-related lipid transfer). START domains are integral components of various signaling proteins in both animals and plants. Mammalian START proteins bind sterols, phospholipids or sphingolipids, and exhibit diverse functions and sub-cellular distribution, including nuclear localization. Despite this there is no reported evidence of their activity in transcription. Moreover, a ligand-binding function has not been confirmed for any START domain proteins from plants. We are using site-directed mutagenesis and deletion of the START domain of GL2 to address its presumed function as a regulatory module. Thus far, two classes of START domain mutants corresponding to highly conserved and/or charged residues within the predicted hydrophobic ligand-binding pocket have been identified. In the first, the mutations are recessive and sensitive to copy number such that the stability of the transcription factor appears affected. An example is the $gl2^{R384L}$ mutant, which corresponds to $StAR^{R182L}$, a mutation affecting a conserved arginine residue in the *StAR* gene from lipoid congenital adrenal hyperplasia (CAH) patients. In the second, the mutations are dominant negative, consistent with the hypothesis that ligand binding but not leucine zipper-mediated dimerization is affected. This is exemplified by $gl2^{K338L}$, which corresponds to a residue implicated in ligand specificity. The sub-cellular partitioning of GL2 wild-type and mutant transcription factors is currently being assessed *in vivo* using fusions to EYFP. Preliminary results suggest that mutations in the START domain alter nuclear localization and perturb the spatial expression pattern of GL2. Our experiments test a model in which the transcriptional activity of HD-ZIP proteins is regulated by a positive feedback loop via ligand binding to its START domain.

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Publications

Carlsson, J., Lagercrantz, U., Sundström, J., Teixeira, R., Wellmer, F., Meyerowitz, E.M. and Glimelius, K. (2007) Microarray analysis reveals altered expression of a large number of nuclear genes in developing cytoplasmic male sterile *Brassica napus* flowers. *Plant J.* **49**:452-462.

Gordon, S.P., Heisler, M.G., Reddy, G.V., Ohno, C., Das, P. and Meyerowitz, E.M. (2007) Pattern formation during *de novo* assembly of the *Arabidopsis* shoot meristem. *Development.* In press.

Gor, V., Shapiro, B.E., Jönsson, H., Heisler, M., Reddy, G.V., Meyerowitz, E.M. and Mjolsness, E. (2006) A software architecture for developmental modeling in plants: The computable plant project. In: *Bioinformatics of Genome Regulation and Structure II*, Springer, New York, pp. 345-354.

Haswell, E.S. (2007) MscS-like proteins in plants. *Curr. Topics Membranes* **58**:329-359.

Haswell, E.S. and Meyerowitz, E.M. (2006) MscS-like proteins control plastid size and shape in *Arabidopsis thaliana*. *Curr. Biol.* **16**:1-11.

Jönsson, H., Heisler, M., Shapiro, B.E., Meyerowitz, E.M. and Mjolsness, E. (2006) An auxin-driven polarized transport model for phyllotaxis. *Proc. Natl. Acad. Sci. USA* **103**:1633-1638.

Long, J.A., Ohno, C., Smith, Z.R. and Meyerowitz, E.M. (2006) *TOPLESS* regulates apical embryonic fate in *Arabidopsis*. *Science* **312**:1520-1523.

McAbee, J.M., Hill, T.A., Skinner, D.J., Izhaki, A., Hauser, B.A., Meister, R.J., Reddy, G.V., Meyerowitz, E.M., Bowman, J.L. and Gasser, C.S. (2006) *ABERRANT TESTA SHAPE* encodes a KANADI family member, linking polarity determination to separation and growth of *Arabidopsis* ovule integuments. *Plant J.* **46**:522-531.

Omelianchuk, N.A., Mironova, V.V., Poplavsky, A.S., Pavlov, K.S., Savinskaya, S.A., Podkolodny, N.L., Mjolsness, E.D., Meyerowitz, E.M. and Kolchanov, N.A. (2006) AGNS (*Arabidopsis* GeneNet Supplementary DataBase), Release 3.0. Proc. Fifth Intl. Conf. on Bioinformatics of Genome Regulation and Structure **2**:223-226.

Omelyanchuk, N., Mironova, V., Poplavsky, A., Podkolodny, N., Kolchanov, N., Mjolsness, E. and Meyerowitz, E. (2006) AGNS - A database on expression of *Arabidopsis* genes. In: *Bioinformatics of Genome Regulation and Structure II*, Springer, New York, pp. 433-442.

Ponomaryov, D., Omelianchuk, N.A., Kolchanov, N., Mjolsness, E. and Meyerowitz, E. (2006) Semantically rich ontology of anatomical structure and development for *Arabidopsis thaliana*. L. Proc. Fifth International Conference on Bioinformatics of Genome Regulation and Structure **2**:227-230.

Ponomaryov, D., Omelianchuk, N.A., Mironova, V., Kolchanov, N., Mjolsness, E. and Meyerowitz, E. (2006) A program method of constructing ontology of phenotypic abnormalities for *Arabidopsis thaliana*. Proc. Fifth International Conference on Bioinformatics of Genome Regulation and Structure **2**:231-234.

Reddy, G.V., Gordon, S.P. and Meyerowitz, E.M. (2007) Unravelling developmental dynamics: Transient intervention and live imaging in plants. *Nature Rev. Mol. Cell Biol.* **8**:491-501.

Sieber, P., Wellmer, F., Riechmann, J.L. and Meyerowitz, E.M. (2007) Redundancy and specialization among plant microRNAs: Role of the miR164 family in developmental robustness. *Development* **134**:1051-1060.

Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J.L. and Meyerowitz, E.M. (2006) Genome-wide analysis of gene expression during early *Arabidopsis* flower development. *PLoS Genetics* **7**:1012-1024.

Wolpert, L., Jessell, T., Lawrence, P., Meyerowitz, E.M., Robertson, E. and Smith, J. (2007) *Principles of Development*, Third Edition, Oxford University Press, pp. 551.

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Summary: The Rothenberg group studies the molecular mechanisms that induce and guide the choice of blood stem cells to develop into T lymphocytes. We focus on identifying the transcription factors and signaling events that trigger T-lineage gene expression in an uncommitted precursor cell, and then determining how these work to force the cell to relinquish other developmental options. T-cell development is an illuminating example of a stem cell-based developmental strategy, distinct in many ways from specification of cell types from a fertilized egg. This system provides unique insights due to the excellent distinctions that can be made between developmental intermediates, the ease with which cell fate can be manipulated, and the great number of molecular markers that are available to define every step toward full T-cell identity. The approaches used in the Rothenberg lab are a combination of *in vitro* developmental biology, high-resolution characterization of individual cell developmental states, and molecular genetics of gene regulation. Kinetic dissection of this complex process using *in vitro* differentiation systems and retroviral perturbation make it possible to solve the roles of individual regulatory molecules in successive, highly defined developmental contexts. In addition, the group is investigating the subtle variations in this pathway that may predispose to autoimmunity. Finally, extending the question of developmental regulation further, the group is also seeking clues to the evolutionary origins of both the T and B lymphocyte developmental programs, by analysis of the mechanisms controlling expression of immune genes in basal vertebrates.

Many cell cycles elapse between the beginning of the T-cell differentiation pathway and the cells' firm commitment to stay on that pathway, and this is a fascinating period in which cell fate is "negotiated" by regulatory factor interplay. The interests of the group for several years have focused on the dynamic balance between two transcription factors, PU.1 and GATA-3, and the influences on them from Notch pathway signaling, all of which appear to play central roles in early T-cell development. GATA-3 is crucial for T-cell development from the earliest stages and is used repeatedly at later steps. PU.1 is notable because at a pivotal decision point early in T-cell development, its role switches abruptly from necessary to antagonistic, and it must suddenly be shut off. Notch pathway signals trigger initiation of the T-cell program, and also keep the cells from veering off into other programs during the extended period before they undergo complete commitment to a T-cell fate. Over the past five years, these three molecules have been illuminating probes of the early T-cell development process.

However, there have remained a number of mysteries about the mechanisms that guide cells into the T-cell pathway. T-cell precursors keep on using a great number of regulatory factors inherited from their multipotent precursors, and relatively few factors have been found that are turned on in a fully T cell-specific way. Are we missing any key regulatory players needed to promote the T-cell fate? How are the cells driven forward through irreversible developmental commitment when they are carrying so much stem cell regulatory "baggage?" What are the actual mechanisms that shut off multipotency-associated genes like the one encoding PU.1 and that enable activation of the T-lineage specific genes? Finally, even the positive transcription factor functions needed to support the T-cell program are extremely dose-dependent. We find that even GATA-3, a required positive regulator of T-cell development that is one of the most T-lineage specific factors in its expression, can actually divert the cells away from the T cell pathway if it is expressed at too high a level. This transcription factor dose-dependence may be a general feature of gene networks for stem cell-based development, and the T-lineage program is an excellent showcase in which it can be studied. Essential T-cell transcription factors not only need to be activated and freed of inhibition by competing regulators; they must also be quantitatively regulated to keep their expression level within tight limits. How is this achieved?

In the past year, we have addressed these questions from several new vantages. New clues based on chromatin markings suggest how GATA-3 expression is maintained within such a narrow range of expression levels during the early, sensitive stages of T-cell development. High-resolution analyses of normal and autoimmune-prone T-cell development have shed new light on the order of key regulatory transitions, sharply defining exactly which T-cell properties are already expressed and which are not yet turned on when genes like PU.1 begin to be shut off. *cis*-Regulatory mapping studies are helping us to zero in on the transcription factor binding sites that are critical to cause this correctly timed downregulation of PU.1. Also, a new regulatory candidate

of great interest is the transcription factor Bcl11b, which is one of the first T cell-specific genes to be upregulated. These studies are adding to our ongoing research on the roles of GATA-3 in lineage choice, the developmental origins of diabetes, and molecular clues to the evolution of the vertebrate immune system.

121. Identification of the critical transcriptional regulators of PU.1 directed myeloid lineage conversion of early T cells

Deirdre D. Scripture-Adams, Benjamin Park, Yang Yang

PU.1 is a powerful myeloid promoting factor that is nevertheless expressed strongly during the earliest stages of T-cell development, and is required for T lineage development. Our previous work (see last year's annual report) has shown that perturbation of PU.1 by retroviral overexpression inhibits T-cell survival and development, both by blocking the expression of critical T-cell transcription factors and signaling molecules, and by blocking the expression of stage-specific functional genes such as *Rag*. These PU.1 activities can also divert early T cells to a myeloid-like fate that manifests as a Mac-1+/F4/80+ and MCSFR+ and/or CD11c+ cell-surface phenotype, with a gene expression profile characteristic of myeloid lineage cells. This diversion occurs only in the absence of Notch signaling, and the Notch signaling pathway only protects cells that have passed the T-cell specification stage. These results suggest that thymic Notch signaling imposes a temporal/developmental check on PU.1 activity only in those thymocytes which have begun a program of T specification, while maintaining vulnerability to PU.1 mediated fate determination in earlier T-cell stages, regardless of Notch signaling.

Analyses of PU.1 induced gene expression changes (either in the presence or absence of notch signals) suggest that PU.1 mediated inhibition of Myb, Heb-alt and Gfi-1 could be of particular importance in the implementation of the PU.1 mediated myeloid conversion. We have now co-overexpressed Heb-alt in combination with PU.1 in a DN3-like cell line, and we find that Heb-alt alone is sufficient to reduce the percentage of cells converted to a Mac-1+ phenotype by half. Moreover, the remaining Mac-1+ (PU.1 deviated) population is only present among cells with the highest levels of PU.1. Among cells with the highest Heb-alt expression, the percentage of Mac-1+ deviated cells drops even further, suggesting that the developmental onset of Heb-alt expression may be of paramount importance in counterbalancing PU.1 expression in order to prevent non-T-cell fates during early thymopoiesis. We have sorted these doubly overexpressing cells, and can now compare their gene expression profiles to determine whether the changes seen with PU.1 overexpression are prevented or altered by expression of Heb-alt. We are currently performing similar co-overexpression experiments to determine the importance of Myb inhibition by PU.1, and we will assess the impact of these co-expressed transcription factors on the inhibition of T-cell regulatory genes that is characteristic of the PU.1 mediated lineage diversion.

To further explore the role of PU.1 in early development, we are performing gene knock down experiments using PU.1 specific siRNAs and PU.1-targeting morpholino antisense oligonucleotides. Transfection of the earliest fetal liver precursors with PU.1 specific siRNA results in a delay in T-cell development at both at the DN1 to DN2 transition, and the DN2 to DN3 transition. Using the non-Notch signaling OP9-control culture system, we find that sorted DN2 thymocytes will express Mac-1 after 3-4 days, but that DN2 cells transfected with either PU.1 or Runx 1-specific morpholinos have dramatically reduced Mac-1 expression. In tandem with the above experimental systems, we are now using conditional PU.1 knock-out mice to explore the effects of early loss of endogenous PU.1 on T-cell development.

122. *cis*-Regulatory analysis of the hematopoietic transcription factor PU.1

Mark Zarnegar

Development of committed T cells requires precise regulation of transcription factors to drive forward the T-cell fate, while preventing alternative fate choices. Some factors must be turned on, while others must be silenced. One transcription factor that plays dual roles, contributing to T-cell development, yet also hindering commitment, is PU.1. This ets family transcription factor is needed at the earliest stages of T-cell development (DN1 and DN2), but must be turned off or else the developing pro-T cells become arrested. Too much PU.1 expression can drive the formation of a myeloid phenotype, whereas too little can block T-cell development from occurring. By studying how PU.1 is silenced as T cells become lineage committed (DN3), we hope more fully to understand the entire network of factors driving formation of T cells.

Multiple genome alignments allowed us to locate several non-coding regions upstream of the transcription start site of PU.1, which could potentially contain *cis*-regulatory function. Nine distinct **C**onserved **E**lements, termed CE1-9, have been revealed. DNase hypersensitivity (DHS) and chromatin immunoprecipitation assays (ChIP), techniques that can identify regions of transcription factor accessibility, and thus potential regulatory function, were used to examine the chromatin context surrounding these CEs. The assays revealed regions that are accessible in both PU.1 expressing myeloid cells and in non-expressing pro-T cells (DN3 stage), at -14 kb near CE8-9, while some regions, CE3-CE7, are only open in myeloid cells. DNase HS mapping, previously done by Jing Chen in the lab, also indicated that a region, CE5-CE3, might contain an element with T-cell function. Using our mapping information from the ChIP and DNase HS data, we postulated that region CE8-9 may contain a non-specific regulatory element, while CE3-7 may contain a myeloid-specific enhancer element, and/or a T cell-specific regulatory region.

Various luciferase reporter constructs were designed to examine the potential functional effects of the CEs. When transiently transfected into PU.1 expressing myeloid cells versus non-expressing pro-T cells, our reporter constructs yielded cell type-specific effects. CE5 functions as an enhancer only in myeloid cells while CE4 contains a T-cell silencer region. In contrast to the cell type-specific effects seen with CE5 and CE4, CE9-8 behaves as a general enhancer. We have demonstrated that the T-lineage specific silencer also governs activity of the PU.1 *cis*-regulatory sequences in the context of stably transfected pro-T and myeloid cells.

Precise mapping has now shown that the T-cell silencer in CE4 is bipartite, including a core 120 bp region with a secondary silencing region closely downstream. Scanning mutagenesis, mutating blocks of six bases across this CE4 core region, has recently revealed key sites needed to maintain the element's repressive function. The mutants that disrupt silencing function overlap interesting predicted transcription factor binding sites, including sites for Ets-, Runx-, and possibly Krüppel-type zinc-finger proteins. We are very intrigued by the Runx and Ets sites, as Runx and Ets factors are known to interact at the protein level in lymphocytes, and cooperatively bind DNA. Runx is also known to be a context-dependent activator or repressor of transcription. Electrophoretic mobility shift assays confirm that Runx1 can bind to a site in our CE4 core region, along with other, as of yet unidentified factors. When we knock down Runx1 by cotransfecting antisense morpholinos in our transient transfection system, we inhibit the silencing effect of CE4. Another of our mutants from the scanning mutagenesis may also have uncovered important nucleotides controlling the context-dependent function of Runx1 occupancy. While some of the mutations simply abolish the silencer activity, one of the mutants appears to convert the CE4 core element into an enhancer.

We are continuing to examine various mutations and attempting to identify other factors that may act in combination with Runx1 to control CE4's silencing ability. We are now starting to generate mice that have stable integrations of our reporters, to provide a proper developmental context for looking at how our CEs contribute to PU.1 expression in all cell types *in vivo*.

123. An essential stage-specific role for GATA-3 in early T-cell development

Deirdre D. Scripture-Adams

Gata-3 is a dual zinc-finger transcription factor essential for embryonic development, and is expressed throughout T-cell development from the earliest precursor through the single positive CD4 or CD8 stages of mature thymocytes. It also plays an important post-thymic role in promoting the development of TH2 cells in the peripheral blood. Although loss of *Gata3* results in embryonic lethality at embryonic day 11 (E11), complementation experiments using wildtype and *Gata3* null mice have shown that *Gata3* null cells can contribute to all hematapoietic lineages except the T lineage. Targeting *Gata3* with anti-sense nucleotides led to an arrest at the DN1 stage of T-cell development. Overexpression studies have also pointed to the importance of proper regulation of Gata-3, as excess levels lead to arrest at DN1 in fetal liver precursors, and reduced proliferation at the DN to DP transition. Tight regulation of Gata-3 is clearly important at the level of regulation of multipotency and cell fate determination, as members of the lab have recently shown that overexpression of Gata-3 in early thymocytes can subvert the T lineage program and convert thymocytes to a mast cell fate. Although these results indicate that Gata-3 is present and must be regulated during T-cell development, there is not yet proof of a rigid requirement for Gata-3 at each of the DN stages, nor any fine delineation of its differing role at each stage.

To address these questions at the stage of the earliest T precursor, we have used retroviral expression of shRNA, or directly transfected siRNA, targeted to *Gata3* to show that loss of Gata-3 leads to a delay in progression from DN1 to DN2, and an even stronger block in progression from DN2 to DN3. This DN2 to DN3 delay was also seen when early precursors were cultured as individual clones in which a *Gata3* specific shRNA was expressed. When each of the early DN stages was examined in isolation, Gata-3 was required for transition from DN2 to DN3 and DN3 to DN4, and from DN1 to DN2 to a lesser extent. We have identified a strong requirement for Gata-3 in maintaining viability of the DN1 and DN3 stages, and a lesser requirement for maintaining the viability of DN2 stage cells. While a transgene expressing the anti-apoptotic protein Bcl-2 could partially compensate for the viability lost when Gata-3 is reduced, it could not overcome the identified developmental arrests generated at each of the fetal liver precursor, DN1, DN2, or DN3 cell stages.

Loss of Gata-3 resulted in changes in the gene expression profiles of the impacted cells, and from this we will identify candidate factors, working with, or as antagonists of Gata-3 that might be responsible for the developmental delays seen in Gata-3-inhibited thymocytes. In all of the cell populations thus far examined, PU.1 gene expression increases when Gata-3 is reduced, and as a transcription factor capable of independently diverting thymocytes into the myeloid lineage, this alone might play a role in the delay in T-cell development. Some other observed changes in gene expression were consistent with loss of Gata-3 causing upregulation of PU.1: Moc-1, for instance, which we know from our PU.1 overexpression studies is up-regulated when PU.1 is overexpressed, is also increased when Gata 3 is inhibited. We have also seen decreases in transcription of CD3 and Rag in cells derived from Kit+ FL precursors when Gata-3 is inhibited, which is consistent with inhibition of the T developmental pathway.

We have now confirmed the developmental arrest phenotypes we observed for the DN1 and DN2 stages of T-cell development using retrovirally transduced Cre to delete the *Gata3* gene from cells of conditional knockout mice. In addition, we have been able to use these mice to

generate c-Kit++ precursors that have already excised *Gata3* before starting T-cell development. In these cells, arrest occurs much earlier, and the cells never progress beyond DN1, strongly suggesting that Gata-3 is required for the earliest manifestations of T cell potential.

124. Transcriptional regulation of *Gata3* in early T-cell development

Jingli Zhang

Studies have shown that like other members of the GATA family, the mouse and human *Gata3* genes contain two distinct promoters and alternative first exons that are expressed in a tissue or lineage specific manner. The first exon generated from the distal promoter (1a), which is highly conserved between mouse and human, is ~9.5kb 5' to the first exon transcribed from the proximal promoter (1b). While exon1b is predominantly transcribed in normal mouse thymocytes as a whole, exon1a is predominately transcribed in mouse brain. In later stage of T-cell development, both exon1b and 1a are transcribed in active Th2 cells with the presence of IL-4. However, total thymocyte populations are overwhelmingly dominated by cells beyond the stage of T-lineage commitment, ~85% DP and 4-10% even more mature classes of CD4 SP and CD8 SP cells. Therefore, the *cis*-regulatory elements that drive the earliest *Gata3* expression during the initial stages of T-cell development have remained uncertain.

Using the OP9-DL1 T-cell development culture system, we have grown DN1-4 pro- and pre-T cells from fetal liver hematopoietic stem cell precursors *in vitro*. Analysis of mRNA indicated that only exon 1b is expressed in the DN1-3 stages, while DN4 cells start to upregulate exon 1a transcription in addition to exon 1b transcription. To understand how DN3 thymocytes are programmed to either activate exon1b or silence exon 1a or both, Chromatin Immunoprecipitation (ChIP) was used to study histone modification patterns of the *Gata3* gene locus during early T-cell development. Conserved non-coding sequences (CNSs) in 5' upstream and intronic regions on the *Gata3* locus have been chosen as candidates for Q-PCR studies on ChIP samples. In fetal liver precursor derived DN3 cells, while the promoter of exon1b and introns 1 and 3 have increased acetylation on Histone3 as compared to other regions, in parallel with transcription activation, the promoter of exon1a and a short segment just 3' downstream of exon1a have increased trimethylation on Histone3/Lysine27. These marks suggest full accessibility for the proximal promoter but active repression of the distal promoter. Interestingly, however, another mark usually associated with increased expression, dimethylation on Histone3/Lysine4, has been observed in both regions, with the exon1b region about two-fold higher than the exon1a region. Taken together, these results suggest that while it is silent in DN3 cells (high level of TriMeH3/K27), exon1a is already (or becomes) poised to be transcriptional competent (increased DiMeH3/K4). In order to understand the dynamic change in transcriptional regulation from DN1 to DN3, ChIP studies will be used to study histone modification patterns of GATA3 gene locus in DN1 cells. DNase hypersensitivity assay and reporter assay will be used to identify the *cis*-regulatory elements in these regions. And gel shift assay will be used to find potential T-lineage specific *trans*-regulatory factors of GATA3.

125. Use of obligate transcriptional repressors to study the role of transcription factors in T-cell development

Deirdre D. Scripture-Adams, Jerry G. Kwong, Mark Zarnegar, Sanket S. Acharya

A number of transcription factors are known to be critical for T-cell development, but relatively few of them have fully defined sets of target genes. Even among transcription factors for which affected genes are known, for relatively few is it known whether the transcription factor directly, or indirectly activates or represses the target gene in question. Both positive and negative gene expression effects are seen, but individual transcription factors often have dual activating or repressing activities that are manifested differentially depending on the *cis*-regulatory context. To circumvent this problem, we have constructed obligate repressor fusions of the important T-cell developmental transcription factors PU.1, Gata-2 and Gata-3, using a repressor domain derived from the *Drosophila melanogaster*. Engrailed protein fused to the DNA binding domain of each of the above factors, and we have inserted these into retroviral vectors. These fusion proteins should unconditionally repress gene expression wherever they are targeted, and so by comparison with the effects of the wild-type transcription factors, they should help resolve which activation or repression effects are direct. We have now infected the Adh.2c2 cell line (a thymoma derived DN3-like cell line) with retrovirus carrying either the wildtype or the ENG fusion version of these transcription factors, sorted cell populations to homogeneity after 48 hours of culture, and are analyzing the changes in gene expression that occur by quantitative real time PCR. The interpretation depends both on the effect of the wild-type factor on each target gene, either negative or positive, and on the similarity or difference between the wildtype and obligate repressor effects. A similar effect on expression of a target gene by both wildtype and obligate repressor will suggest that the transcription factor acts directly as a repressor on that target gene or only indirectly as an activator. If the effect on expression of a target gene by the wild-type factor is substantially different from the ENG fusion, this may suggest that the factor in question acts directly as an activator or indirectly as a repressor (e.g., by repressing a repressor). We are now extending our experiments into primary fetal thymocytes, and will use the data generated to assign direct or indirect regulatory status to the genes which have PU.1, Gata-2, or Gata-3 influenced expression patterns. This will greatly assist in the placement and usage of target genes within the gene network controlling T-cell development.

126. Non-homogenity in T-cell developmental progression: Using single-cell culture to look at stochastic and non-stocastic models of developmental stage transition and fate choice

Deirdre Scripture-Adams

In the thymus, T cell precursors do not progress through development in a uniform cohort, even when sorted to apparent homogeneity: Even a single cell can give rise to daughters which have differing rates of progression along a given developmental pathway, or which have made divergent fate choices. The mechanisms behind the fate divergence of daughter cells remain unclear. We are using fetal liver precursors to begin to address these questions by sorting individual precursors directly on to supportive stromal layers and culturing them while monitoring expansion, divergence, and progression along the T pathway. Our preliminary results show that daughter cells of fetal liver precursors progress to DN1 (29% of clones), DN2 (11% of clones), DN3 (22% of clones) and DN4 (39% of clones) when cultured for a two-week period on OP9 stromal cells expressing the Notch ligand DL1. Of these clones, 30% were found to contain non-T lineage cells (these were predominantly putative myeloid lineage with mac-1 expression). The likelihood of having non-T lineage cells was most associated with cells progressing only to the DN1 stage (44%), then the DN4 stage (33%), followed by DN2 (11%) and DN3 (0%). We are using single cell colonization experiments assayed multiple times over a given culture period to quantify differing precursor frequencies, as well as differing tendencies toward alternative non-T fate choice as a starting point from which to begin to model the mechanisms underlying daughter cell fate variances.

127. Defining transitional stages during T lineage specification and commitment

Mary Yui, Jonathan Moore, Ni Feng

The T cell differentiation program is initiated upon entry of precursor cells into the Notch ligand-rich thymus. During the earliest CD4 and CD8 double negative (DN) stages of development the cells undergo rapid proliferation while turning on the T cell specification program. In progressing from early thymic precursor (ETP) to DN2 and then DN3 stages, cells become increasingly committed to the T cell lineage, losing the ability to take on different non-T cell fates. The DN2 stage in particular is critical to the understanding of T cell specification and commitment, as these cells have been shown to turn on T cell identity genes and downregulate non-T cell genes, while still permitting development to alternative fates, such as dendritic cells, macrophages, natural killer (NK) cells, and mast cells. In studying the gene expression changes occurring during the DN1 and DN2 stages of T-cell development, using high expression of c-Kit (CD117, stem cell factor receptor) as a stringent criterion in addition to the CD44 and CD25 used traditionally, improves the purification of the DN1 subset, separating the true early thymic precursor (ETP) population from contaminating mature NK and T cells. Furthermore, we have found that using c-Kit levels we are able to separate the DN2 population into discrete developmental stages, one with very high levels of c-Kit, DN2a, and the other with slightly lower c-Kit and CD44, DN2b. The DN2a subset appears to represent the earliest stage of true T-lineage specification.

Using these newly defined populations we are now conducting a detailed gene expression analysis using quantitative real time PCR on purified T cell receptor-negative subsets, ETP, DN2a, DN2b, and DN3a. This has revealed a surprising number of changes between DN2a and DN2b, underlining the importance of this distinction. We found that ETP cells express very few T cell identity genes, although *Gata3* and several Notch target genes such as *Hes1* are already relatively high in this population. Between ETP and DN2a two transcription factors (currently being studied in our group) are dramatically upregulated, *Bcl11b* and an alternative isoform of *Tcf12* (HEBalt), and some T cell identity genes have just begun to be turned on. By DN2b the T cell identity genes, including *Cd3e* and *Ptcra* (preTα) have been dramatically upregulated, although most do not reach their maximum levels of expression until the DN3a stage. Genes required for alternative lineages, including *Sfpi1* (Pu.1) and *Tal1* (SCL), decline from DN2a to DN2b and further by DN3a.

Comparing development of DN2a and DN2b cells in OP9-DL1 culture over a 10-day period, DN2b cells generate fewer cells overall but more mature populations, which include intermediate single positive (ISP) and CD4 and CD8 double positive (DP) cells, while DN2a cell numbers are higher but >90% of cells still retain the DN phenotype. These populations may also have differing developmental potentials in terms of non-T cell fates and αβ-versus γδ-T cell fates. This division of the DN2 stage will allow us to more precisely define the genetic programming that determines proliferation, differentiation and fate choices of early T cells.

128. A unique lineage-specific regulatory factor in early T-cell development: Bcl11b

C. Chace Tydell, E.-S. David-Fung[*]*, Tom Taghon*[**]

Through an intensive gene discovery project, we have identified a transcription factor, Bcl11b, to be the product of the gene that is most dramatically upregulated during the T-lineage specification process. Although suspected of possible tumor suppressor function, little is known about its role in these early stages of intrathymic T-cell development.

The gene discovery project was designed to identify genes specifically upregulated during early T-cell development as a source of new candidates for regulatory function that had not previously associated with T-cell differentiation. This gene discovery project used a pro-T cell cDNA probe depleted of sequences shared with premyeloid and multipotent precursor cells to probe a mammalian cDNA macroarray library, described in previous Annual Reports. Exploring the genes captured by the screen in greater detail, we were particularly excited

about the dramatic upregulation of a putative transcription factor, Bcl11b, at the time of T-lymphocyte specification. This gene stood out as the only regulatory gene found in this screen, or in a companion screen in our lab, which undergoes lineage-specific upregulation of >50 fold during this critical transition. Bcl11b (originally named for its sequence homology to B-cell lymphoma protein 11a) was reported in 2003 to be a tumor suppressor gene in T-cell lymphomas. Bcl11b has been implicated in a later stage of T-cell development, at the checkpoint between the DN3 stage and all later TCRαβ T-cell development, but the basis of this effect and any earlier role at the time of the first Bcl11b induction are not known.

The regulatory context in which Bcl11b is upregulated is largely unaltered from the state of prethymic, multipotent hematopoietic progenitors. The cells are still expressing a full complement of "legacy" factors at similar levels to multipotent precursors or stem cells and have not yet upregulated most T-cell genes. Bcl11b upregulation coincides with the transition from DN1 (or ETP) to DN2 stage *in vitro*, as well as *in vivo*, at a time point that depends precisely on the initial developmental status of the precursors. Thus, it is tightly coupled to specification and not a side effect of signals from a particular thymic microenvironment nor an artifact of particular culture conditions. Unlike almost all other T-lineage regulatory genes, Bcl11b reaches its peak level of expression almost immediately and then is expressed continuously throughout T-cell maturation. It is the best existing regulatory candidate for a T-lineage "identity" factor.

We now have a colony of mice with a flox'd Bcl11b gene (a gift of Mark Leid of Oregon State University) that we are beginning to use to investigate the effects on early development of conditionally knocking out Bcl11b gene expression. For example, progenitors with excised Bcl11b could develop normally towards a B-cell fate in the OP9-control co-cultures. In the absence of Bcl11b, it is expected that αβ-T-cell development will halt in the OP9-DL1 co-cultures, but what will happen to these cells? It is feasible that the early DN populations will ignore available Notch signal and become B cells or some other alternative cell type (dendritic cells or natural killer cells). Alternatively they may pause in their development, they may die or they might become γδ-T cells.

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129. Molecular analysis of T-lineage commitment: The role and regulation of Bcl11b

Long Li

Hematopoietic stem cells (HSC) commit to the T-lineage by gradually losing developmental plasticity and gaining T-cell identity. A recently discovered transcription factor, Bcl11b, could be a key regulator in this process, based on its expression pattern (see preceding two abstracts). In hematopoietic tissue, the expression of Bcl11b is restricted to the T lineage. Its mRNA level increases dramatically once HSCs commit their fate to T lineage. Loss-of-function studies have revealed that Bcl11b is essential for TCRαβ T-cell development, though peculiarly not for the development of TCRγδ T cells, which also express it. We have found that its expression is roughly opposite to that of PU.1, which represents the potential to differentiate into B and myeloid lineages, and to other stem-cell "non-T" regulatory factors such as SCL, Id2 and GATA2. However, Bcl11b is apparently a repressor, not an activator, because it interacts directly with histone deacetylases. Based on this evidence, it is rational to speculate that Bcl11b could be one gene for which we have been looking that stimulates T-cell development by inhibiting developmental plasticity of T-cell precursors. The objective of this project is to address the role of Bcl11b and also to determine the mechanism that turns it on in T-cell development.

Bcl11b is subject to alternative splicing, and distinct biological functions could map to alternatively spliced exons. To study these effects, different Bcl11b cDNAs were cloned into a MIGR1 retroviral vector, and fetal liver HSCs infected with retroviral Bcl11b were co-cultured with OP9-Delta1 (OP9-DL1) bone marrow stromal cells to support T-cell development or OP9 control cells to support non-T cell fates. One prevalent isoform of Bcl11b, consisting of exons 1-2-4, severely inhibited proliferation in T-lineage differentiation cultures when we used retroviral vectors to express it precociously in hematopoietic precursors. Even when cells transgenic for the antiapoptotic protein Bcl2 were used in these experiments, proliferation was consistently inhibited, although T-lineage differentiation could be detected. However, alternative splicing products yield different results, with hints that at least one isoform can actively promote entry into a DN2-like stage even in the absence of appropriate Notch signaling. A critical question is also how the effects of Bcl11b differ from those of its relative, Bcl11a, which is expressed in a reciprocal pattern. We are therefore studying the functional role of Bcl11a, as well as all four of the transcripts of Bcl11b we have detected (Bcl11b 1-2-4, 1-3-4, 1-4 and 1-2-3-4), in early T-cell development by using retroviral gene delivery.

The expression pattern of Bcl11b is unique, because it is the only gene we have found so far with such extreme T-lineage and developmental stage-specific expression. Understanding of how Bcl11b is regulated will dramatically increase our knowledge of T-lineage commitment. Therefore, in parallel with the retroviral transduction studies, we have so far mapped DNA methylation in flanking *cis*-regulatory regions of *Bcl11b* by using bisulfite DNA sequencing of CpG islands. Apparently, there is a single unmethylated window that we have been able to define in a conserved noncoding region in Bcl11b-expressing P2C2 cells, a line of pro-T cells, which is fully methylated in myeloid hematopoietic cell lines. We are going to map additional chromatin markings and use these as a guide to locate the biologically relevant

transcription factor binding sites that regulate the expression of Bcl11b in T lineage.

130. Determining the role of Suz12 in early T lymphocyte development

Harold Hsu, Long Li

Polycomb group proteins (PcG) are essential for development. It is now known that the PcG proteins are required to maintain the transcriptionally repressive state of many important development regulators. PcG proteins act by forming multiprotein complexes: polycomb repressive complex (PRC) 1, 2, and 3. Suz12 (suppressor of zeste 12, a zinc-finger DNA binding protein) is a component of PRC2, which includes the histone H3-K27 methyltransferase EZH2 and chromatin remodeling protein RbAp48. The PRC2 also directly interacts with DNA methyltransferases to repress transcription. A recent study has mapped Suz12 binding sites across the entire non-repeat genome as they are found in human embryonic stem cells. The results showed that Suz12 occupies regulatory regions of ~1,800 genes, and that most of the genes are tissue-specific genes. Notably, these target genes include the powerful B-lineage regulator *Pax5* and the subject of the two previous abstracts, *Bcl11b*. The finding suggests that Suz12 and PRC2, the complex it belongs to, may actively restrain the developmental potential of embryonic stem cells.

Interestingly, in mice, there is a unusually high expression of Suz12 in the thymus, which indicates that this gene may play a role in T-cell development. Based on this information, the objective of this project is to investigate the effect of knockdown of Suz12 expression in pro-T cells on early T-cell development. Courtesy of Dr. Tian Chi of the Yale School of Medicine, we have obtained a miRNA vector (LMP-Suz12) that specifically targets Suz12 mRNA.

We are using the OP9-DL1 and OP9-control co-culture systems to test whether Suz12 specifically influences progression through T-cell developmental stages or the irreversibility of lineage commitment.

131. Early T-cell development and αβ vs. γδ lineage choice in non-obese diabetic (NOD) mice

Mary Yui, Ni Feng

The NOD mouse is a model for autoimmune Type 1 diabetes. This disease in humans, as well as NOD mice, is polygenic and dependent upon the pathogenic and protective responses of various T-cell populations to self-antigens. All T-cell lineages (e.g., CD4, CD8, αβ, γδ, NKT, Treg) arise from the same precursor cells in the thymus, which undergo proliferation, differentiation, lineage choices, and selection for self-tolerance before maturation and export to peripheral tissues. We have previously found several defects in early T-cell development of NOD mice, which may have an impact on T-cell selection, immune responsiveness, and/or autoimmunity. One of the traits, a violation of the first major T-cell checkpoint at β-selection, appears to map to at least two diabetes susceptibility loci based upon preliminary data. We have also found that early T-cell populations from NOD mice exhibit differences in expression levels of several cell surface receptors, which may play a role the β-selection checkpoint violation. Furthermore, we have found abnormalities in the selection or survival of αβ T cells at the $CD4^+CD8^+$ double-positive (DP) cell stage. This NOD trait is seen in the OP9-DL1 coculture system, which normally allows the development and survival of precursor cells to the DP cell stage. Very few DP cells develop or accumulate from NOD early T-cell precursors. This failure of differentiation may be revealed primarily by the co-culture system; however, we have also noted that a higher-than-normal proportion of BrdU-staining cells disappear *in vivo* between the DN and DP stages, during a period of rapid proliferation. In addition, a higher proportion of γδT cells arise from the same early T populations from NOD mice when cultured on OP9-DL stromal cells. The γδT cells from NOD mice appear to undergo more proliferation than those from normal C57BL/6 mice, but it is not yet clear whether these cells also differentiate preferentially into the γδT lineage because of an intrinsic alteration in TCR signal strength. We will continue to investigate the differentiation, proliferation and survival of αβ- and γδ-T cells generated from NOD early T cells *in vitro* and *in vivo*.

132. Gene expression analysis of early T-cell subsets from non-obese diabetic (NOD) mice

Chen Yee Liaw, Mary Yui, Ni Feng

NOD mice exhibit several defects in early T-cell development, which may have an impact on T cell-dependent autoimmune Type 1 diabetes. One such trait is a loss of the β-selection checkpoint control in NOD mice that are unable to rearrange a T cell receptor (TCR) due to deficiency of a recombinase gene, *Rag1*, or loss of a DNA repair enzyme, *Prkdc* (scid) (Yui and Rothenberg, 2004). To investigate the molecular basis of this checkpoint violation phenotype, the early T-cell developmental programming was compared between NOD.*Rag*$^{-/-}$ and normal B6.*Rag*$^{-/-}$ mice by analysis of cell surface receptors and gene expression. RNA was extracted from sorted early T-cell subsets (CD4 and CD8 double negative, DN1-3 cells) from NOD and B6 mice in the presence or absence of a *Rag*-deficiency and reverse transcribed to cDNA. Real-time quantitative PCR was performed on these samples for approximately 40 genes. These genes include those involved in T-cell identity (e.g., *Cd3e, Ptcra)*, transcription factors (e.g., *Gata3, Tcf7*), Notch signal targets (e.g., *Dtx1, Hes1*), and other genes known to be expressed differentially during development. Most of the T-cell identity genes and key T-cell transcription factors were expressed at similar levels between comparable NOD.*Rag*$^{-/-}$ and B6.*Rag*$^{-/-}$ cell subsets (as well as between NOD and B6 wild-type cell populations), suggesting that development proceeds fairly normally in NOD mice and that these populations can be considered comparable. Few consistent differences in gene

expression were seen between the two mouse strains. However, a few genes were expressed at lower levels in specific NOD populations than in their counterparts from B6 mice. These genes include *Tcf12* (encoding an alternative isoform of HEB), a Signaling Lymphocyte Activation Molecule (SLAM) receptor, CD150, and a tyrosine-specific phosphatase that downmodulates TCR signaling, *Ptprj* (CD148). None of these genes is known to play a role specifically in β-selection, but both of the latter genes have known functions in modulating TCR signaling in mature T cells. In addition, abnormal populations found in NOD.*Rag*$^{-/-}$ thymuses, after β-selection breakthrough, were purified and their gene expression patterns determined. Several of these populations show unusual combinations of expressed genes, suggesting a derangement in developmental programming. One of these populations may be early breakthrough cells or may be otherwise involved in the breakthrough phenotype. We plan to analyze additional samples and other genes to try to determine the progression of the checkpoint breakthrough.

Reference

Yui, M.A. and Rothenberg, E.V. (2004) *J. Immunol.* **173**(9):5381-5391.

133. Analysis of TCR signal sensitivity in early T cells from NOD.*Rag*$^{-/-}$ mice

Mary Yui, Ni Feng, Chen Yee Liaw

The spontaneous loss of β-selection checkpoint control in NOD.*Rag*$^{-/-}$ mice at 6-8 weeks of age leads to a breakthrough of double-negative (DN) cells to CD4 and CD8 double-positive (DP) cells, in the absence of any T-cell receptor (TCR) rearrangement, the normal trigger for passing through this checkpoint. This breakthrough is likely to arise from a spontaneous partial T-cell receptor (TCR) signal generated aberrantly by the NOD.*Rag*$^{-/-}$ DN cells. To investigate the possibility that NOD DN cells have a greater TCR signal sensitivity which might contribute to the β-selection breakthrough or other known defects in NOD T-cell functions, NOD.*Rag*$^{-/-}$ and B6.*Rag*$^{-/-}$ mice, under 6 weeks of age, were injected with graded doses of anti-CD3 antibodies, which mimic TCR signaling and stimulate DN cell differentiation to DP cells. At lower anti-CD3 doses, differentiation to DP appears to be fairly similar between the two mouse strains, however, a higher proportion of NOD.*Rag*$^{-/-}$ cells responded to the highest dose as measured by upregulation of a cell surface marker, CD5. The CD5 receptor, which is expressed in proportion to the strength of signal, is not only expressed at higher levels in the responding NOD.*Rag*$^{-/-}$ cells, but is expressed in a higher percentage of cells. Moreover, very few cells remain in the DN compartment after anti-CD3 injection of NOD.*Rag*$^{-/-}$ mice. While this result indicates that thymocytes from NOD.*Rag*$^{-/-}$ mice may be more sensitive to a strong anti-CD3 TCR stimulation, these studies need to be confirmed. In addition, we are attempting to recapitulate the system *in vitro* on OP9-DL stromal cells so the doses, environmental signals, and the specific cell populations can be more easily controlled and experimentally manipulated.

134. Regulatory analysis of lamprey recombinant antigen receptor genes

Jonathan E. Moore

How the highly complex vertebrate adaptive immune arose is as yet a largely unexplained evolutionary question. The adaptive immune systems of all the jawed vertebrates are, to first order, the same; however, there is little evidence of a jawed-vertebrate-like adaptive immune system in the invertebrates. This implies that this complex system must have been evolved in a relatively short time, 50 to 100 million years, and this has been called an evolutionary "big bang."

Over the last few years, this big bang has become less mysterious. For example, RAG-1 and RAG-2 have recently been discovered in the sea urchin; previously the closest sequence relatives to the jawed vertebrate RAGs were bacterial. Another example more relevant to this project involves lampreys. Lampreys have the same physiological responses to infection and skin grafts as their sister taxa, the jawed vertebrates. However, they do not have recombinant antibodies, recombinant T-cell receptors, MHC, RAGs, or a thymus. Until three years ago, there was little hint of a way out of this conundrum; then, Zeev Pancer, Chris Amemiya, and Max Cooper announced the discovery of a different recombinant molecule, the variable lymphocyte receptor or VLR, expressed on the surface of lamprey lymphocyte-like cells.

Previous work in our lab had shown that the transcription factors involved in immunological regulation are largely conserved within the jawed vertebrates. If these very different adaptive immune systems of lampreys and the jawed vertebrates share a common origin, then it would be a reasonable hypothesis for these same transcription factors to be involved in the immunological regulation of lamprey immune genes. To this end, we are attempting to determine the transcription factors regulating the lamprey VLR.

We are using a three-pronged approach for this. First are bioinformatic assays, which include the transcription factor binding site program MatInspector and a number of comparative and clustering analysis programs of our own design. These have predicted several interesting regions, two of which have yielded interesting results with other methods.

The second method utilizes heterologous transfection reporter assays. From these, we have found a 150 bp region up-regulates transcription 6- to 10-fold in catfish T- and B-cell lines. In addition, we have found another region of several hundred bp which up-regulates transcription 6-fold in a murine B-cell line. We plan to use a scanning mutagenesis approach to refine the regions responsible for this effect.

The third method uses binding assays to identify sites engaged by transcription factors that may correlate with functional expression in different cell types. This is important for comparison between lamprey VLR+ blood

cells, which cannot be transfected, and defined blood cell types of jawed vertebrates that can be transfected. With nuclear extracts from the catfish cells listed above, we have recently scanned the above-mentioned 150 bp region for binding, and have found several 25- to 30-bp probes which are bound. We have nuclear extracts from sorted VLR+ and VLR- lamprey cells, and we are now carrying out similar assays using these extracts.

135. Bioinformatic tool design and application: Clues to generation of diversity in lamprey recombinant antigen receptors

Jonathan Moore

In the field of bioinformatics, there is an increasingly great need for methods to deal with diverse medium-scale experimental data. These methods fall in the "nether world" more complicated than a few simple BLAST searches or primer designs and yet less extensive than a vertebrate genome assembly or genome-wide microarray analysis. To fill a part of this void, we have developed convenient algorithms for noise reduction, statistical tests, comparative sequence analysis, and data display.

Among these tools, the quantitation of transcription factor site density over medium-scale sequences has been extremely useful as a guide to construct design for VLR gene regulation. While our main interest is on the cell type-specific factors that drive transcription of VLR genes as a whole, the most important function of these genes depends on their unique mechanism for recombinational generation of diversity. It is clear that the recognition sequences used are not the same as those used by Rag1/Rag2 recombinases for jawed vertebrate immunoglobulin and TCR genes. Therefore, we are extremely interested that the bioinformatic approaches have also shown a high density of transcription factor binding sites near the two sites of the VLR gene where recombination occurs. These factors which bind these sites are likely responsible for the generation of the VLR's diversity. To determine the nature of these factors, I have begun cloning these regions for reporter and protein-binding assays.

Publications

Rothenberg, E.V. (2007) Negotiation of the T-lineage fate decision by transcription factor interplay and microenvironmental signals. *Immunity* **26**:690-702.

Rothenberg, E.V. (2007) Regulatory factors for initial T-lymphocyte lineage specification. *Curr. Opin. Hematol.* **14**:322-329.

Rothenberg, E.V. (2007) Cell lineage regulators in B and T-cell development. *Nat. Immunol.* **8**:441-444.

Rothenberg, E.V. and Yui, M.A. Development of T cells. In: *Fundamental Immunology, 6th Edition* W.E. Paul, (ed.), Lippincott, Williams & Wilkins, Chapter 13. In press.

Taghon, T., Yui, M.A. and Rothenberg, E.V. (2007) Mast cell lineage diversion of T-lineage precursor cells by the essential T-cell transcription factor GATA-3. *Nat. Immunol.* **8**:845-855.

Tydell, C.C., David-Fung, E.-S., Moore, J.E., Rowen, L., Taghon, T. and Rothenberg, E.V. (2007) Molecular dissection of prethymic progenitor entry into the T-lymphocyte developmental pathway. *J. Immunol.* **179**:421-438.

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Summary: During this past year we have continued work with the Alliance for Cellular Signaling and developed a number of collaborations to focus on the role of G protein-mediated circuitry in white cell function, both in macrophages and in other immune system related functions.

In addition we have continued our work in collaboration with Drs. David Anderson and Allan Basbaum trying to understand the function of G protein-mediated signaling pathways in small diameter nociceptor neurons that innervate the skin. Dr. Sang-Kyou Han has been exploring the effect of a variety of mutations in mice that we and others have generated that effect components of the signaling mechanism in small diameter nociceptive neurons. He found that PLCβ3-deficient mice lose the ability to respond to agonists of the histamine H1 receptor that are injected intradermally. Normally the mice respond by scratching and Dr. Han has identified a subset of small diameter nociceptive neurons that in wild-type mice have PLCβ3 and also have histamine H1 receptors and that may be responsible for this form of the "itch" response. In the absence of PLCβ3 these mice no longer respond. He has also found a series of other agonists that induce "itch" responses, some of them requiring PLCβ3 and others that function in the absence of PLCβ3. Current work is focused on the analysis of these different "itch"-inducing systems and their relationship to the cells and systems that transduce "pain" responses. We are also continuing to study the relationship of the PLCβ3 deficiencies and other mutations including the effects of the loss of function of P2X3 an ATP mediated ionotropic receptor.

Two other projects are ongoing; one involves a computational study of the two component signaling systems in microorganisms. A very large and diverse family of these signal transducing molecules have been discovered through metagenomic sequencing. Another collaboration with Steve Quake and Robert Bao involves the development and application of microfluidic devices to study the nature of "noise" in the GPCR signaling circuits in both tissue culture cell lines and in primary cells.

136. Molecular Biology Laboratory of the Alliance for Cellular Signaling (AfCS)

Iain Fraser, Joelle Zavzavadjian, Leah Santat, Jamie Liu, Estelle Wall, Mi Sook Chang, Baljinder Randhawa, Xiaocui Zhu, Melvin I. Simon

In their normal physiological environment, mammalian cells are exposed to a wide range of ligands to which they can respond. In the presence of multiple stimuli and the consequent activation of multiple signaling pathways, how does the cell integrate these inputs to produce a specific response? The Alliance for Cellular Signaling (AfCS) conducted a large-scale ligand screen in the mouse macrophage-like cell line RAW264.7 to assess crosstalk between simultaneously activated signaling pathways. Recent analysis of this dataset suggests that pathway crosstalk is not irreducibly complex and could be mediated by 'interaction agents' [1]. Our current research is focused on identification of interaction agents that mediate crosstalk between macrophage signaling pathways activated through G-protein-coupled receptors and toll-like receptors. One of our projects attempts to identify a mechanistic basis for the anti-inflammatory action of cAMP on macrophage activation. Use of sophisticated vector-based RNAi approaches has confirmed PKA as the effector through which PGE2 modulates a subset of LPS-induced cytokines. A variety of approaches, including phosphoprotein profiling, RNAi and yeast 2-hybrid screens, suggest that modulation of a component of the NFκB family could be the mechanism through which cAMP/PKA can attenuate LPS-induced pro-inflammatory cytokine expression. A further project focuses on the mechanism through which certain Ca^{2+}-inducing GPCR ligands synergize to produce robust Ca^{2+} responses in macrophages, which can enhance the innate immune response. AfCS studies have shown that Ca^{2+} synergy induced by C5a and UDP requires select members of the PLC β family. We have hypothesized that this selective use of certain isoforms may relate to binding of PDZ domain-containing scaffolding proteins to the C-termini of the PLC beta3 and PLC beta2 proteins. We have identified a PDZ family protein that binds specifically to these isoforms and we are currently investigating whether this protein is required for Ca^{2+} synergy in C5a+UDP activated macrophages. We have also published reports this year on a large-scale reagent resource developed and distributed by our group [2], and a plasmid-based platform for multi-gene RNAi [3].

References

1. Natarajan, M., Lin, K.M., Hsueh, R.C., Sternweis, P.C. and Ranganathan, R. (2006) *Nature Cell Biol.* **8**:571-580.

2. Zavzavadjian, J.R., Couture, S., Park, W.S., Whalen, J., Lyon, S., Lee, G., Fung, E., Mi, Q., Liu, J., Wall, E., Santat, L., Dhandapani, K., Kivork, C., Driver, A., Zhu, X., Chang, M.S., Randhawa, B., Gehrig, E., Bryan, H., Verghese, M., Maer, A., Saunders, B., Ning, Y., Subramaniam, S., Meyer, T., Simon, M.I., O'Rourke, N., Chandy, G. and Fraser, I.D. (2007) *Mol. Cell Proteo.* **6**:413-424.
3. Zhu, X., Santat, L.A., Chang, M., Liu, J., Zavzavadjian, J.R., Wall, E.A., Kivork, C., Simon, M.I. and Fraser, I.D. (2007) *BMC Mol. Biol.* **8**:98.

137. PLCβ3 mediates both itch and pain through the activation of the 5-HT2A receptor

Sang-Kyou Han, Valeria Mancino, Hee-Ju Kim, Melvin I. Simon

Serotonin (5-HT) is a potent proinflammatory and noxious agent, which causes hyperalgesia as well as an itch sensation in rodents. Although DRG neurons express multiple serotonin receptors, the activation of the 5-HT2A receptor was required to elicit scratching responses when injected intradermally into the rostral back skin. These drugs produced the thermal hyperalgesic response, but not the scratching reaction when they were injected into the hind paw. We found that both scratching activities and hyperalgesic responses were almost completely abolished in PLCb3$^{-/-}$ mice, suggesting that the 5-HT2A receptor coupled to PLCβ3 is responsible for mediating the serotonin-induced itch sensation and the hyperalgesic response.

These results showed that the 5-HT2A-PLCβ3 signaling cascade is primarily involved in eliciting itch at the thoracic DRG and pain at the lumbar DRG. At least two possibilities can be suggested regarding the circuitry underlying itch and pain processing. One is that serotonin can excite different subsets of DRG neurons depending on spinal level (e.g., L4, L5 DRG Vs a given level of thoracic DRG) with different connectivity to spinal dorsal horn neurons. The other possibility is that serotonin may excite the same subset of neurons, but differing in central connectivity. To test these hypotheses, as a first step, we are investigating whether serotonin or a-methyl-5HT-induced calcium responses are attributable to the action of PLCβ3 expressed in C-fiber nociceptive neurons. Next, using double *in situ* analysis combined with various molecular markers, we will investigate whether DRG neurons expressing both PLCβ3 and 5-HT2A receptor belong to a particular subgroup of DRG neurons, and whether localization of this subset is changed at different levels of spinal cord. To determine the connectivity to spinal dorsal horn neurons whose response properties are eliminated in PLCβ3 KO mice, we will use serotonin (or α-Methyl 5-HT)-induced c-Fos expression and follow this analysis with an electrophysiological analysis of the properties of dorsal horn neurons in different segmental levels (cervical C3-C6 dorsal horn vs. L4-5 dorsal horn). The spinal neurons showing PLCβ3-dependent c-Fos activity may reflect itch and hyperalgesic input depending on injection site. Accordingly, these results will provide critical evidence as to how itch and pain pathways form their specific neural circuitry in dorsal horn neurons.

138. Attenuation of allergic contact dermatitis through the PLCβ3 signaling

Sang-Kyou Han, Hee-Ju Kim, Valeria Mancino, Melvin I. Simon

Allergic contact dermatitis affects about 5% of men and 11% of women in industrialized countries and is one of the leading causes of occupational diseases. The disease is characterized by a loss of immunological tolerance toward small allergenic molecules. The allergen first penetrates the epidermis and is taken up by skin dendritic cells, which migrate to the draining lymph nodes and present haptenated peptides to antigen-specific T lympocytes. Previously, we found that PLCβ3 signaling is an important mediator of the acute itch sensation. In this study, we have tested whether this signaling molecule is involved in signaling allergic contact dermatitis. To address this question, we investigated cutaneous contact hypersensitivity in PLCβ3 KO mice and their littermates using the contact allergens such as DNCB, DNFB and oxazolone, which generate selective cutaneous T-cell-mediated allergic responses upon repeated allergen contact. In murine systems, repeated application of these haptens has been used widely as a model of chronic allergic contact dermatitis. Using repeated application of DNCB or DNFB onto dorsal back skin, we created a chronic contact dermatitis model in PLCβ3+/+ and PLCβ3-/- mice. Interestingly, we found that erythma or skin ulceration induced by repeated application of these haptens in PLCβ3-/- mice appeared earlier than in PLCβ3+/+ mice. Compared to wild-type littermates, PLCβ3 KO mice treated with DNCB or DNFB showed exaggerated allergic responses. Our histological examination revealed that the epidermis was at least two-fold thicker in PLCβ3 KO mice than in their wild-type littermates after the third application of DNFB. The level of IgE, allergic immunoglobulin, was greatly enhanced by 5-fold in PLCβ3KO mice after exposure to DNCB. Toluidin blue staining on ulcerated skin sections also revealed that the number of mast cells in the PLCβ3-/-skin was increased by 3.5-fold compared to wild-type littermates after the third application of DNCB or DNFB, respectively. These results indicated that PLCβ3 acts to attenuate allergic contact dermatitis. Currently, we are investigating the molecular mechanisms underlying allergic contact dermatitis at the cellular and subcellular level.

139. Does ATP mediate sensory function between keratinocytes and C-fiber nociceptors?

Sang-Kyou Han, Valeria Mancino, Hee-Ju Kim, and Melvin I. Simon

Most of c-fiber nociceptors, particularly $IB4^+$ neurons, are known to innervate the epidermal layer in the skin and most of these nerve fibers have polymodal responses to both temperature and mechanosensation. In addition, keratinocytes were found to respond to warmth sensation and have been suggested to act as mechanosensors. These phenomena suggested the possibility that there is communication between keratinocytes and nerve fibers to mediate these sensations. It has been suggested that keratinocytes might release ATP as a signaling substance to which $IB4^+$C-fibers respond. However, it is much more complicated because DRG neurons contain more than 10 different ATP receptors, ionotropic P2Xs and metabotropic P2Y receptors. Therefore, multiple ATP receptors expressed in given cells may compensate for each other in gene deletion experiments designed to identify the sensory function contributed by ATP. To overcome this difficulty, we generated P2X3PLCβ3 double KO mice because P2X3 is a major isoform expressed in $IB4^+$ neuron, and PLCβ3 may couple to various P2Y receptors. So, by eliminating ATP responses in $IB4^+$ DRG neurons, we want to determine what modality can be mediated by ATP. As expected, ATP responses were significantly decreased in $IB4^+$ neurons derived from the double KO mice, but not much affected in $IB4^-$ neurons. Currently, we are investigating the behavioral consequences of these $P2X3^{-/-}PLC\beta3^{-/-}$-deficient mice.

140. A microfluidic platform for cell signaling assays

Xiaoyan R. Bao

We have built and tested an experimental platform for probing calcium responses in mammalian cells within microfluidic devices. Using pressure-actuated microvalves, we can precisely gate and switch flow to cells anchored via fibronectin to floors of separate microchannels; conditions within the microchannels are tightly regulated and analogous to those in standard cell culture incubators. The channels are arranged next to each other so that many different experiments can be imaged in a microscope at the same time. In each experiment, calcium responses in about a hundred individual cells can be imaged through the device using standard epifluorescence microscopy. Analysis of variations across cellular populations and channel averages indicate that variability between experiments are within a factor of two of the lower limit set by cell-to-cell variations. Finally, in addition to calcium imaging, we can also perform differential experiments on NF-κB translocation in response to stimulation via Toll-like receptors.

We have two ongoing experiments taking advantage of the perfusion control and single cell sensitivity afforded by our platform. The first one is to correlate, on a cell-to-cell level, calcium release in response to G-protein-coupled receptor activation. These experiments seek to track down the source of cell-to-cell variations in cellular responses to G proteins. The second experiment is to use differential perfusion timing to understand the kinetics of receptor desensitization.

Publications

Han, S.-K, Mancino, V. and Simon, M.I. (2006) Phospholipase Cb3 mediates the scratching response activated by the histamine H1 receptor on C-fiber nociceptive neurons. *Neuron* **52**:691-703.

Natarajan, M., Lin, K.M., Hsueh, R.C., Sternweis, P.C. and Ranganathan, R, (2006) A global analysis of cross-talk in a mammalian cellular signalling network. *Nature Cell Biol.* **8**:571-580.

Pero, R.S., Borchers, M.T., Spicher, K., Ochkur, S.I., Sikora, L., Rao, S.P., Abdala-Valencia, H., O'Neill, K.R., Shen, H., McGarry, M.P., Lee, N.A., Cook-Mills, J.M., Sriramarao, P., Simon, M.I., Birnbaumer, L. and James, J.J.L. (2007) G alpha(i2)-mediated signaling events in the endotheliurn are involved in controlling leukocyte extravasation. *Proc. Natl. Acad. Sci. USA* **104**:4371-4376.

Shin, K.-J., Wall, E.A., Zavzavadjian, J.R., Santat, L.A., Liu, J., Hwang, J.-I., Rebres, R., Roach, T., Seaman, W., Simon, M.I. and Fraser, I.D.C. (2006) A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression. *Proc. Natl. Acad. Sci. USA* **103**:13759-13764.

Simon, M.I., Crane, B. and Crane, A. (eds.) Methods in Enzymology, Volume 422, Two-component systems, Part A. San Diego, Academic Press/Elsevier, 2007.

Simon, M.I., B. Crane, B. and Crane, A. (eds.) Methods in Enzymology, Volume 423, Two-component systems, Part B. San Diego, Academic Press/Elsevier, 2007.

Zavzavadjian, J.R., Couture, S., Park, W.S., Whalen, J., Lyon, S., Lee, G., Fung, E., Mi, Q., Liu, J., Wall, E., Santat, L., Dhandapani, K., Kivork, C., Driver, A., Zhu, X., Chang, M.S., Randhawa, B., Gehrig, E., Bryan, H., Verghese, M., Maer, A., Saunders, B., Ning, Y., Subramaniam, S., Meyer, T., Simon, M.I, O'Rourke, N., Chandy, G. and Fraser, I.D. (2007) The alliance for cellular signaling plasmid collection: A flexible resource for protein localization studies and signaling pathway analysis. *Mol. Cell Proteo.* **6**:413-424.

Zhu, X., Chang, M.S., Hsueh, R.C., Taussig, R., Smith, K.D., Simon, M.I. and Choi, S. (2006) Dual ligand stimulation of RAW 254.7 cells uncovers feedback mechanisms that regulate TLR-mediated gene expression. *J. Immun.* **177**:4299-4310.

Zhu, X., Santat, L.A., Chang, M., Liu, J., Zavzavadjian, J.R., Wall, E.A., Kivork, C., Simon, M.I. and Fraser, I.D., (2007) A versatile approach to multiple gene RNA interference using microRNA-based short hairpin RNAs. *BMC Mol. Biol.* Under review.

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Summary: There are currently three major areas of investigation in the laboratory: the understanding of *cis*-regulatory mechanisms controlling patterning of cells and how genes function in a network to guide developmental processes; the integration of imaging and mathematical modeling to test hypothesis regarding how organisms sense size; and the analysis of coordinated cell movement during migration of cells.

Gene-regulatory networks controlling embryonic development

Using expression data and genetic and enhancer analyses to identify targets of the Dorsal transcription factor, we have generated a preliminary gene regulatory network (GRN) that describes how ~60 genes interact during gastrulation and subsequent differentiation in *Drosophila*. Together they form a regulatory network controlling gastrulation and contributing to homeostasis and adaptive responses at later stages.

We have identified ~300 genes that are expressed along the dorsoventral axis of *Drosophila* early embryos. Enhancers have been identified for 30 genes, the most known for any developmental process to date, and comparative studies of these enhancers provide evidence that a "*cis*-regulatory code" exists to effect differential expression. Using this information, we have generated a preliminary gene network that describes the interactions among ~60 genes, and which begins to describe regulatory interactions responsible for controlling cell movement and differentiation during gastrulation. We propose to define the function of all genes expressed along the dorsoventral axis of *Drosophila* embryos in order to decipher the underlying logic of the Dorsal GRN.

Our goals are to develop a complete understanding of how transcription factors control gene expression, and to identify genes regulating processes such as cell migration and differentiation whose misregulation leads to disease. We have extended these studies to later timepoints during development to analyze specification of mesoderm derivatives. Our ultimate goal is to achieve "developmental reengineering" of fruit flies, so that similar studies in vertebrates are possible.

Self-organization and scaling of dorsal-ventral patterning in *Drosophila* embryos

One of the most striking properties of some developing systems is the ability to re-organize their developmental program and apparently give rise to normal adults when the size or shape of the embryo is altered. This phenomenon has fascinated several generations of embryologists and remains as a central problem in developmental biology.

We are currently using genetic and computational approaches to understand how patterning conveyed by morphogen gradients scales with size. In the early *Drosophila* embryo, the NFκB-like maternal factor Dorsal (dl) has a central role in organizing patterning along the dorsal-ventral axis. dl is a transcription factor ubiquitously localized in the early embryo, but this protein enters the nucleus only where the Toll receptor is activated. Although, dl is not a classical morphogen, because production of the protein does not stem from a localized source, the nuclear expression of dl forms a gradient that determines the fate of cells along the dorsal-ventral axis in a concentration dependent manner. Since nuclear dl provides the first coordinates of positional information in the embryo, the ability to generate a well-proportioned fly might depend on whether or not patterning by dl – and transcription factors downstream of it – scale to the size of the dorsal-ventral axis.

Cell movement coordination during migration of cells

Gastrulation is the process by which cell movements lead to the specification of different tissue types. In *Drosophila melanogaster*, three phases of gastrulation encompass highly coordinated movements of the presumptive mesoderm within the developing embryo. As a result, cells acquire different cellular environments that trigger activation of signaling pathways to differentially effect tissue specification. Many genes involved in the process of cell migration have been described, but, surprisingly, the mechanisms by which cells communicate to move directionally in a coordinated fashion remain unclear.

We aim to understand the coordination of cell movement during migration of cells, a process that makes essential contributions to embryonic development and homeostasis. We hypothesize that during cell migration "leader cells" are defined by localized signals received from neighboring cells and that these "leaders" guide the entire population of migrating cells towards a defined location. Furthermore, we propose that migrating cells exhibit differential activation of signaling pathway(s) within the population that coordinates *en masse* movement. We will use the *Drosophila* model system to

gain insights into this important process, capitalizing on the recent sequencing of its genome and our own work allowing *in vivo* imaging of all cells within a developing embryo.

141. Characterization of the protein localization of Pyramus and Thisbe, *Drosophila* FGF ligands

Sarah Payne, Angelike Stathopoulos

Fibroblast Growth Factors (FGFs) are involved in important developmental processes including mesoderm induction and patterning, organ formation, and neuronal differentiation. A pressing question in the FGF field is how specificity is achieved and distinct cellular responses accomplished with so few receptors compared to ligands. The FGF family of ligands has two relatively new members in *Drosophila*, Pyramus (Pyr) and Thisbe (Ths), both of which activate the Heartless (Htl) FGF receptor. This 2:1 ratio of Pyr and Ths ligands to their receptor, Htl, provides a simplified model of the situation in vertebrates. We use this model to investigate how signaling by Pyr versus Ths could give rise to different cellular responses. We made HA-tagged versions of Pyr and Ths proteins and have shown these proteins to be functional in the embryo. These epitope tags have allowed us to visualize the proteins using GAL4 lines to overexpress them in the mesoderm and neuroectoderm. These patterns will be compared with those in an *htl* mutant background to see if the ligand-receptor complex is required to stabilize the protein. Further investigation of the proteins in a *pyr/ths* deficiency mutant background will allow us to see whether the protein localization of one ligand is affected when the other is not present.

142. Identification of Dorsal-dependent enhancers functioning in the *Drosophila* early embryo

Anil Ozdemir, Manoj Samanta, Angelike Stathopoulos

During early *Drosophila* development, embryonic cells are destined to specific cell fates as a result of responses to multiple signaling pathways. What defines specification of these cells into individual types and how multiple signaling pathways contribute to differentiation have long been remained unclear. The maternal transcription factor Dorsal is distributed in a gradient along the dorsal-ventral axis of *Drosophila* blastoderm, and is responsible for the expression of early zygotic genes. Multiple transcriptional thresholds that are created by the Dorsal gradient initiate formation of germ layers in *Drosophila* embryo. Classical genetic screens, expression profiling experiments and computational methods have identified many key targets of the Dorsal gradient network. The significant amount of data available however is still not enough to explain the underlying signaling networks that control the dorsal-ventral patterning of the *Drosophila* embryo. To elucidate *in vivo* target genes and *cis*-regulatory elements of Dorsal, Twist and Snail, we performed chromatin immunoprecipitation followed by hybridization to DNA chip (ChIP-chip) experiments by using antibodies directed against each transcription factor. Analysis of ChIP-chip data identified genes that are already known to be targets of Dorsal-Twist-Snail gene network. Interestingly, new enhancers of the known target genes were also identified. Most importantly, ChIP-chip revealed around 100 novel targets of Dorsal-Twist-Snail gene regulatory network. To test whether identified regions bound by Dorsal, Twist and/or Snail of known or newly identified target genes can act as *cis*-regulatory sequences, we cloned a representative number of these DNA regions in lacZ/GFP reporter constructs and injected them into embryos. Current analysis of the transgenic fly lines for the expression pattern of the reporter genes will shed light on mechanisms of transcriptional regulation during early development. To gain insight into the function of the novel target genes during early *Drosophila* morphogenesis, we are trying to knockdown a representative set of these genes by injecting *ds*RNA into the embryos. *In vivo* live imaging of *ds*RNA-injected embryos will help us understand the function of the newly identified targets during dorsal-ventral patterning in *Drosophila*.

143. Mechanisms for scaling in dorsal-ventral patterning in the early *Drosophila* embryo

Marcos Nahmad, Angelike Stathopoulos

One of the first discovered and most surprising abilities of some developing systems is that of regeneration. For example, several animals are able to re-organize their developmental program and give rise of aparent normal adults when part of the embryo is removed. This suggests the existence of an unclear mechanism for scaling that strongly correlates patterning and size. In the early *Drosophila* embryo, the maternal factor Dorsal is responsible for organizing patterning along the dorsal-ventral axis. An ectopic Dorsal gradient generated along the anteroposterior axis in embryos that lack the endogenous dorsal-ventral patterning is sufficient to reproduce all kinds of transcriptional outputs (Huang *et al*., 1997). This system provides a good opportunity to study possible mechanisms of size-dependent scaling in development. We show that the expression patterns of some Dorsal target genes scale with respect to the length of the axis. For example, the position of the gene *vnd* is very similar in both mutants and wild-type embryos, and the length of the domain of expression scales with the length of the axis. We suspect that the mechanism responsible for patterning may involve a distinct-opposing gradient. We are currently identifying and studying, both experimentally and mathematically, the existence and properties of such an opposing gradient. This study may provide an explanation to the general mechanisms underlying scaling and self-organization during embryonic patterning.

144. Weckle is a DNA-binding protein that regulates dorsoventral patterning of *Drosophila* embryos

Snehalata Kadam, Phoebe Tzou, Louisa Liberman, Desirea Mecenas, Angelike Stathopoulos

Patterning along the dorsoventral axis of *Drosophila* embryos is controlled by the Rel transcription factor, Dorsal (dl). dl is present in a nuclear gradient. The highest level of nuclear dl, in ventral regions, activates genes such as *sna* and *twi* (Type I response), whereas lower levels of nuclear dl present in ventrolateral regions supports expression of other genes like *rho* and *vnd* (Type II). The lowest levels of nuclear dl are present in dorsolateral regions and support the expression of genes *sog* and *thisbe* (Type III). These distinct outputs are determined in part by dl binding site affinity and also by combinatorial interactions between dl and other transcriptional activators (i.e., Twist). Definition of the Type III response remains more elusive, as to date no other transcription factor that might function together with dl in defining this response has been identified.

We have characterized a Zn-finger containing protein, Weckle (Wek), and determined that it functions to regulate dorsoventral patterning together with dl. We have found that Wek protein is localized both to the plasma membrane and to the nucleus. In addition, we have demonstrated that Wek is a DNA-binding protein that recognizes a consensus DNA-binding sequence that often overlaps with a dl binding site. The dorsalized cuticular phenotype of embryos obtained from *wek* germline clone females using hypomorphic *wek* alleles has been previously described [Luschnig *et al.* (2004) *Genetics* **167**:325]. We have determined embryos derived from germline clones using these *wek* alleles exhibit specific defects in dorsoventral axis patterning. Dorsal-dependent threshold outputs Type I and II are completely absent and Type III responses are defective in *wek* mutant embryos. To ascertain whether the *wek* is also required for Type III responses, we have created *wek* null mutant alleles using recombination methods; characterization of these mutants is currently in progress. We propose that Wek functions together with dl to directly specify distinct outputs of gene expression.

145. The role of localized repressors in *Drosophila* dorsal ventral patterning

Mayra Garcia, Angelike Stathopoulos

The *Drosophila* pre-gastrula embryo is patterned by a nuclear gradient of the transcription factor Dorsal, which sets the boundaries of the presumptive mesoderm, neurogenic ectoderm, and non-neurogenic ectoderm. Targets in the neurogenic ectoderm have high affinity Dorsal binding sites and can be activated by both high and intermediate levels of Dorsal but are repressed in ventral regions by other Dorsal targets. Targets expressed in ventral regions have low affinity Dorsal sites and can only be activated by the highest levels of Dorsal. Current models postulate that limiting amounts of Dorsal delineate the dorsal borders of the target genes, although recent *cis*-regulatory analysis and the sharpness of the borders suggest that this may not be the case. It is still unclear how the dorsal borders of Dorsal target genes are established. Our goal is to show that localized repressors set the dorsal borders of Dorsal target genes. This will change our current understanding of how the dorsal-ventral axis of the *Drosophila* embryo is patterned. Previous synthetic enhancer analysis of the *intermediate neuroblast defective (ind)* enhancer, located a short 12 base pair repetitive sequence that mediates repression in dorsal regions, when in the context of 100 base pairs. We conducted further analysis of this element and found that it is sufficient to mediate repression in dorsal regions. We believe that a repressor, which is expressed in dorsal regions, binds to this sequence and sets the dorsal border of *ind* and possibly other Dorsal target genes. We are currently working on a yeast-one-hybrid screen to identify the protein that binds to this element. Once we identify the protein we plan to make mutants if they do not already exist and characterize the gene. We have also initiated the analysis of various *cis*-regulatory regions to search for other repressor binding sites.

146. Neurogenic ectoderm patterning and specification within *Drosophila* embryos

Louisa Liberman, Tigran Bacarian[*], Eric Mjolsness[*], Angelike Stathopoulos

We are interested in determining how the neurogenic ectoderm is patterned in the *Drosophila melanogaster* embryo. The NF-kappa-B like transcription factor, Dorsal, forms a morphogen gradient that has been implicated in the specification of mesoderm, neuroectoderm and dorsal ectoderm in a concentration-dependent fashion. We have developed a quantitative imaging technique to determine the quantity of Dorsal protein present along the axis. This method uses confocal image stacks to make a 3D rendering of the embryo. We show that the Dorsal nuclear intensity decreases approximately five-fold across the dorsalventral axis. In addition to quantitative imaging we are performing a *cis*-regulatory analysis of the binding sites in the enhancers of Dorsal targets to determine which components are necessary and sufficient to drive expression in a broad lateral strip in the developing embryo. It is unclear how the embryo uses an analog input (Dorsal gradient) to produce quasi-digital outputs in the form of gene expression domains with discrete borders and remarkable reproducibility. *cis*-Regulatory analysis suggests transcription factors function cooperatively, binding regulatory sites with varying degrees of affinity; this specificity, in addition to the order and number of sites, may govern threshold responses.

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147. **On the interpretation of morphogen gradients: A lesson from Hedgehog signaling in the *Drosophila* wing**

Marcos Nahmad, John Doyle, Angelike Stathopoulos

Morphogen gradients provide concentration-dependent positional information in several developing systems. Although the mechanisms of action of morphogen gradients in development have been recently discussed extensively, it remains unclear how cells exposed to an extracellular gradient interpret different concentrations to target distinct cellular responses. Using a data based mathematical model, we study how the Hedgehog (Hh) morphogen is interpreted in the developing wing of the fruitfly *Drosophila melanogaster*. A steady state analysis, reveals the existence of a direct nonlinear relationship between the concentration of extracellular Hh and the concentration of the activated form of Smoothened (Smo), which functions as the transducer of Hh signaling. We show that the Hh concentration gradient is interpreted into a step-like response of active Smo. This result suggests that cells may accurately distinguish between on and off states of Hh signaling but cannot explain how the Hh gradient at steady-state can induce more than two different spatial expression domains in a concentration-dependent manner. An alternative mechanism, referred as time integration, is that cells differentially respond to a temporal exposure of a constant concentration of the signal. Although, studies in vertebrates have suggested this possibility, it is unclear how cells can measure their temporal exposure to Hh (Yang *et al*., 1997; Harfe *et al*., 2004). We propose a molecular model for time integration in Hh signaling based on the dynamics of Smo phosphorylation. We are currently validating these predictions *in vivo* and *in vitro*.

148. **Live imaging of gastrulating *Drosophila* embryos using 2-photon microscopy**

Amy McMahon, Willy Supatto, Scott E. Fraser[*]*, Angelike Stathopoulos*

Gastrulation is a conserved yet highly complicated embryonic process combining cell migration and morphological changes, which ultimately results in the establishment of different germ layers. The three phases of gastrulation in *Drosophila melanogaster* consist of the invagination of cells on the ventral surface of embryos, an epithelial to mesechymal transition (EMT), and the spreading of these cells to form a monolayer. These events encompass highly coordinated movements of the presumptive mesoderm within the developing embryo. The gene networks involved in this process have been extensively studied, but surprisingly the details regarding how these genes interact to control this process remain unclear. In addition, previous studies of gastrulation utilized sections of fixed embryos, thus severely limiting the analysis of this highly dynamic and rapid process. The fibroblast growth factor (FGF) receptor, Heartless (Htl), is thought to be required for migration, but whether the migration is actually abnormal or just delayed in *htl* mutants is indeterminate by examination of fixed embryos. We contend that live imaging of developing *Drosophila* embryos will provide insights into the mechanisms used by cells to follow proper paths of migration. Using 2-photon microscopy, we plan to examine fluorescently labeled mesodermal cells in the *Drosophila* embryo and to analyze their migration computationally. In addition, we will carry out a screen to find genes involved in migration and perform live imaging studies of mutants we isolate. Using these tools, we will determine which factors influence cell migratory behavior and help to establish whether there is an underlying migratory program that acts in many cell types.

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Publication

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Summary: We seek to understand how the genome controls the development, behavior and physiology of *C. elegans*. We use molecular genetics to understand detailed mechanisms, and functional genomics to obtain global views of development and behavior. We take computational approaches to understand signal transduction, developmental pattern formation and behavioral circuits. We try to couple tightly computation and experimental data, in part to use computation to make experimental tests more efficient. Moreover, we study other genomes, genetics, and biology of other nematodes to help us comprehend *C. elegans*, to learn how development and behavior evolve, and to learn how to control parasitic and pestilent nematodes.

In the area of signal transduction, we continue to define pathway interactions and to understand the determinants of signaling specificity: how does the same pathway lead to distinct outcomes in different tissues? For these studies we analyze EGF-receptor signaling, WNT signaling, and G-protein-mediated signaling pathways. Last year, we had discovered a new role for EGF signaling, controlling behavioral quiescence (a sleep-like state) during nematode development. This year, we found that the EGF-receptor acts in a single neuron, ALA, during this process, and acts via a phospholipase-C-gamma-mediated diacylglycerol-signaling pathway.

Vulval development involves a remarkable series of intercellular signaling events that coordinate the patterning of the uterine and vulval epithelia and allow them to connect precisely. Specification of the anchor cell from the ventral uterine epithelium breaks the symmetry of the gonad. The anchor cell then produces the vulval-inducing signal, LIN-3, an epidermal growth factor-like protein that acts via *C. elegans* homologs of EGF-receptor, RAS and MAP kinase. Inductive signaling is regulated at the level of ligand production as well as the responsiveness of the receiving cells. LIN-3 is produced in a highly localized and regulator manner. After the anchor cell induces the vulva, a complex program of further pattern formation, cell type specification and morphogenesis follows. The primary (1°) vulval lineage generates an E-F-F-E pattern of cell types while the 2° vulval lineage generates an A-B-C-D pattern of cell types. We now have our hands on a number of receptor proteins, transcription factors and regulated genes; we are trying to define this regulatory network to understand how organogenesis is genetically programmed. For example, we found that the Tailless-type nuclear hormone receptor NHR-67 acts with other factors to regulated cell type-specific gene expression. The anchor cell recognizes one of the seven vulval cell types and invades the vulval epithelium in a process akin to tumor metastasis, and we have found genes necessary for this process. Regulation by the EGF-receptor, WNT and HOM-C pathways impinge not only on vulval development but also the neuroectoblast P12 specification, male hook and spicule development. By comparing these examples with vulval development, we seek to understand the signaling specificity and signal integration. The relative contributions of EGF and WNT differ in each example. Multipotent cells with three fates utilize NOTCH signaling as well as EGF/WNT signaling.

Our efforts in genomics are experimental and computational. Our experimental genome annotation includes identifying *in vitro* binding sites for transcription factors, testing enhancer function in transgenic worms, and systematic inactivation of *C. elegans* transcription factors. We are investigating ways to compare the genomes of *Caenorhabditis* species. In particular, we are collaborating with the Genome Sequencing Center of Washington University to annotate new nematode genomes. Our computational projects involve establishing pipelines for *cis*-regulatory computational analysis, new programs to use orthology and known binding sites or motifs, etc. We have successfully combined information from worms, flies and yeast to predict gene-gene interactions in *C. elegans*, and are extending this to other species. This predicted network was used to interpret the relationship among genes expressed in a nematode that parasitizes sheep.

We are part of the WormBase Consortium, which develops and maintains WormBase, a web-accessible comprehensive database of the genome, genetics and biology of *C. elegans* and close relatives (www.wormbase.org). We initiated WormBook, an open-access online text of *C. elegans* biology associated with WormBase. We have developed Textpresso (www.textpresso.org), an ontology-based search engine for

full text of biological papers. Textpresso is used by *C. elegans* researchers, as well as the WormBase staff; we have made versions for *Neuroscience* (www.textpresso.org/neuroscience) as part of the Neuroscience Information Network) and *Drosophila* (in collaboration with FlyBase (www flybase.org). We are also part of the Gene Ontology Consortium (www.geneontology.org), which seeks to annotate gene and protein function with a standardized, organized vocabulary.

Our behavioral studies focus on understanding male mating behavior as well as locomotion of both sexes. For specific projects we study egg laying, feeding, chemotaxis, osmotic avoidance, among other simple behaviors. Mating behavior, with its multiple steps, is arguably the most complex of *C. elegans* behaviors. Because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, male mating is useful to elucidate how genes control behavior. We are studying several aspects of male mating behavior to understand the neuronal circuits that control the behavior and how they are genetically encoded. Our comparative studies include both analyzing behavioral differences among species, and genetic analysis of *C. briggsae*, *Pristionchus pacificus* (a nematode species we discovered during the 1990s), and *Heterorhabditis bacteriophora* (an insect-killing nematode). We discovered that *H. bacteriophora* uses the same sensory neuron as *C. elegans* to respond to cues to exit the infective juvenile or dauer larval stage.

We have developed a machine-vision system that automatically quantifies the locomotion of nematodes. We use this system to study individual genes, to examine epistatic interactions among genes, and to obtain data to support mathematical modeling efforts. We are expanding this effort to analyze mating behavior of nematodes. We are involved in efforts to model worm movement, vulval pattern formation (in collaboration with Anand Asthagiri in Chemical Engineering, Caltech) and aspects of signal transduction.

149. Nutritional control of larval development: DAF-16 is required for transcription of *cki-1* and repression of *lin-4* during L1 arrest

Ryan Baugh

C. elegans larvae initiate post-embryonic development only in the presence of food, although they can survive starvation for weeks in a developmentally arrested state (L1 arrest). In contrast to the well-studied dauer arrest, L1 arrest occurs without morphological modification, although larvae in L1 arrest are more resistant to environmental stress than developing larvae. Consistent with its role in dauer formation and aging, others and we have shown that insulin/insulin-like growth factor (IGF) signaling regulates L1 arrest [Baugh and Sternberg, *Curr. Biol.* (2006); Fukuyama *et al*. *Curr. Biol.* (2006); Kao *et al*., *Cell* (2007)]. Our results indicate that *daf-2* insulin/IGF receptor mutants have a constitutive L1 arrest phenotype when fed and extended survival of L1 arrest when starved. Conversely, daf-16/FOXO mutants have a defective arrest phenotype, failing to arrest development and dying rapidly when starved. We have shown that DAF-16 is required for transcription of the cyclin-dependent kinase inhibitor *cki-1* in lateral epidermal seam cells in response to starvation, accounting for the failure of *daf-16* mutants to arrest somatic cell division during L1 arrest. Other developmental events such as cell migration, cell fusion, and expression of the microRNA *lin-4*, a temporal regulator of post-embryonic development, are also observed in starved *daf-16* mutants. These and other results implicate insulin/IGF signaling in the nutritional control of development during the first larval stage in *C. elegans*, although additional DAF-16-independent pathways must also participate. In addition to performing a genome-wide characterization of mRNA expression during L1 arrest, we are currently investigating the insulin ligands and their sites of action as well as additional candidate signaling pathways that regulate L1 growth and arrest in response to changing environmental conditions.

150. *C. elegans* EVI1 proto-oncogene, EGL-43, is necessary for notch-mediated cell fate specification and regulates cell invasion

Byung Hwang, Alejandro Meruelo[*]

During *C. elegans* development, *lin-12* (Notch) signaling specifies the anchor cell (AC) and ventral uterine precursor cell (VU) fates from two equivalent pre-AC/pre-VU cells in the hermaphrodite gonad. Once specified, the AC induces patterned proliferation of vulva via expression of *lin-3* (EGF) and then invades into the vulval epithelium. Although these cellular processes are essential for the proper organogenesis of vulva and appear to be temporally regulated, the mechanisms that coordinate the processes are not well understood. We computationally identified *egl-43* as a gene likely to be expressed in the pre-AC/pre-VU cells and the AC, based on the presence of an enhancer element similar to the one that transcribes *lin-3* in the same cells. Genetic epistasis analyses reveal that *egl-43* acts downstream of or parallel to *lin-12* in AC/VU cell fate specification at an early developmental stage, and functions downstream of *fos-1* as well as upstream of *zmp-1* and *him-4* to regulate AC invasion at a later developmental stage.

Characterization of the *egl-43* regulatory region suggests that *egl-43* is a direct target of *lin-12* and *hlh*-2 (E12/47), which is required for the specification of the VU fate during AC/VU specification. egl-43 also regulates basement membrane breakdown during AC invasion through a FOS-1-responsive regulatory element that drives *egl-43* expression in the AC and VU cells at the later stage. Thus, *egl-43* integrates temporally distinct upstream regulatory events and helps program cell fate specification and cell invasion.

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151. EGF signaling specificity

Adeline Seah

In *C. elegans*, LET-23(EGFR) activates two signal transduction pathways: RAS-MAPK for P11/P12 cell fate determination, vulval development, larval viability and male spicule development and IP3-Ca2+ for ovulation. It is not known what determines the specificity among the Ras-MAPK-dependent inductions. One possibility is that different transcription factors act in parallel to or downstream of the EGF signal in different tissues. Hox genes have been implicated as one class of regulatory elements that confer specificity to the EGF pathway in P11/P12 cell fate determination and vulva development. Another possibility is that different signaling pathways interact to generate specificity. Together with the EGF pathway, the Wnt signaling pathway regulates both P11/P12 specification and vulva development as well. Therefore, identifying tissue-specific downstream targets of EGF signaling as well as how different signaling pathways act with the EGF pathway in various tissues will be useful in determining how specificity is generated. We are studying the role of the EGF and Wnt pathways in regulating Hox gene expression and fate specification in spicule development and in male hook development, where we have identified a new role for EGF signaling. EGF signaling has been shown to be required for the specification of certain B lineage fates (g, a and e) in the spicule precursor cells. In both male tissues, equivalent groups of cells are present that are able to adopt the fates specified by the EGF pathway or the Wnt pathway or both. Our results suggest that EGF signaling, but not Wnt signaling, is required for Hox *ceh-13::GFP* expression in a spicule precursor cell, Bgamma. Wnt signaling, however, is required to orient the plane of division of Bgamma. In addition, decreased EGF signaling in *lin-17*/WNT-R mutants enhances their hook defects, while constitutive activation of EGF signaling can partially suppress the hook defects of these mutants.

152. EGF and behavioral quiescence

Cheryl Van Buskirk

The epidermal growth factor receptor (EGFR)/ErbB receptor tyrosine kinases regulate several aspects of development, including the development of the mammalian nervous system. ErbB signaling also has physiological effects on neuronal function, with influences on synaptic plasticity and daily cycles of activity. However, little is known about the effectors of EGFR activation in neurons. Here we show that EGF signaling has a nondevelopmental effect on behavior in *Caenorhabditis elegans*. Ectopic expression of the EGF-like ligand LIN-3 at any stage induces a reversible cessation of feeding and locomotion. These effects are mediated by neuronal LET-23 and phospholipase C-gamma (PLC-gamma), diacylglycerol-binding proteins, and regulators of synaptic vesicle release. Activation of EGFR within a single neuron, ALA, is sufficient to induce a quiescent state. This pathway modulates the cessation of pharyngeal pumping and locomotion that normally occurs during the lethargus period that precedes larval molting. EGF promotes sleep in rodents, and thus our results reveal an evolutionarily conserved role for EGF signaling in the regulation of behavioral quiescence.

153. POPTOP and polarity: Three branches of Wnt signaling contribute to tissue polarity during *C. elegans* organogenesis

Jennifer L Green, Takao Inoue

Analysis of how Wnts govern tissue polarity *in vivo* is limited by our current knowledge of transcriptional targets. To avoid this obstacle we made POPTOP, an *in vivo* TOPFLASH-like reporter of canonical Wnt pathway activity in *C. elegans*. We used a combination of POPTOP expression analysis and genetic analysis to study the controlled orientation of asymmetric cell division in the *C. elegans* vulval epithelium, a classic model for organogenesis. Vulval development requires the cells composing the posterior half of the vulva (P7.p lineage) to reverse their planar polarity such that their organization is a mirror reflection of the cells in the anterior half (P5.p lineage). Wnt receptors LIN-17/Frizzled and LIN-18/Ryk act in parallel to reverse the polarity of the vulval precursor cell (VPC), P7.p, in response to LIN-44/Wnt and MOM-2/Wnt. Our data suggest that EGL-20/Wnt acts via a third parallel pathway to establish or maintain basal uniform anterior-posterior polarity in the field of VPCs and that LIN-17 and LIN-18 reverse this basal polarity in P7.p and reinforce this basal polarity in P5.p. In support of this model, we found that reducing the activity of multiple Wnts at the time of VPC orientation results in a randomization of both P5.p and P7.p orientations. Examination of POPTOP expression yields mechanistic insight by revealing that LIN-17 and LIN-18 activate canonical Wnt pathways in P5.p and P7.p and also reveals involvement of a non-canonical Wnt/MAPK pathway. Thus, although multiple Wnts are required for proper patterning of the epithelium, they do not function redundantly but instead specifically activate distinct signaling pathways.

154. Efficient identification of highly conserved *cis*-regulatory elements in the *ceh-13/lin-39* Hox cluster using *Caenorhabditis brenneri* and *n. sp.* PS1010

Steven Kuntz, Erich Schwarz, John DeModena, Tristan De Buysscher[1], Diane Trout[1], Paul W. Sternberg, Barbara Wold[2]

To accelerate genome-wide studies of transcriptional regulation during development, it is critical to determine in nematodes how much more efficiently phylogenetic footprinting with N-way comparative genomics can identify *cis*-regulatory elements than uninformed promoter dissection. Through exhaustive informatic and transgenic analysis, we wanted to identify what *cis*-regulatory elements control Hox cluster genes and determine if they are conserved across distant phyla with vertebrates. The *ceh-13/lin-39* Hox clusters of *C. elegans*,

C. briggsae, and *C. remanei* were computationally scanned for ungapped sequence similarity with our sequencing of the corresponding *Caenorhabditis spp*. CB5161 and PS1010 Hox clusters. 2% of the 27 kb non-coding sequence was identified as highly conserved evolutionarily by MUSSA, a tool for finding ungapped blocks conserved between multiple genome sequences. The entire non-coding sequence was divided into ten highly conserved regions -- each consisting of a conserved site and flanking sequence -- and nine poorly conserved regions. 90% of the highly conserved regions drove unique *in vivo* expression patterns (six novel and three previously identified elements), representing a four-fold increase over the poorly conserved regions, of which only 22% drove detectable expression (one novel and one previously identified element). This suggests that nematode *cis*-regulatory elements are strongly enriched in or near conservation sites. The five most highly conserved putative *cis*-regulatory elements have been mutated to experimentally assay their expression activity *in vivo*. So far, the conserved site from one of these putative *cis*-regulatory elements has yielded a position-specific frequency matrix (PSFM) that we refined by two cycles of first scanning the *C. elegans*, *C. briggsae*, and *C. remanei* genomes for additional sites with ³80% match to the consensus PSFM, and by then including matches in an enhanced PSFM. This consensus showed statistically significant enrichment in the three genomes over genome-wide searches with 100 different scrambled versions of the same PSFM. Surprisingly, some of these elements have potential copies that are highly conserved in the HoxA clusters of several different vertebrates. We are currently determining the prevalence and locations of these elements in vertebrate genomes, and testing whether mouse Hox sequences containing them drive the same expression pattern in *C. elegans* as the orthologous nematode elements.

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155. *cis*-Regulatory analysis of four *Caenorhabditis* genomes

Erich Schwarz, Ali Mortazavi[1], Steven Kuntz, Alok Saldanha, Bruce Shapiro[2], Barbara Wold[3], Paul W. Sternberg

We have scanned four *Caenorhabditis* genomes (*C. elegans*, *C. briggsae*, *C. remanei*, and the newly sequenced *C. brenneri*) for conserved non-coding DNA elements that could indicate *cis*-regulatory signals. Pilot analyses of the *ceh-13/lin-39* Hox cluster with MUSSA, a tool for finding ungapped blocks conserved between multiple genomic sequences, showed that experimentally validated regulatory elements closely coincide with such blocks in non-protein-coding DNA. We thus, used the open-source Cistematic framework to scan the entire *C. brenneri* genome for such elements. In our first analysis, Cistematic used a fast derivative of MUSSA to find conserved blocks of non-coding DNA in the flanks and introns of ~10,000 genes with single orthologs in *C. brenneri*, *C. brenneri*, and *C. brenneri*. Conserved blocks were scanned with MEME, to identify up to 10 statistically overrepresented position-specific frequency matrices (PSFMs) per gene. This gave ~61K raw PSFMs. Two rounds of genome-wide rescanning, phylogenetic filtering, and merging of highly similar PSFMs yielded 16,385 refined PSFMs; these were checked for statistically exceptional associations with 5,100 gene-specific biological conditions such as Gene Ontology terms, anatomical expression patterns, and homology groups. We found 1,045 PSFMs showing such associations with 265 conditions, including broad categories such as "nervous system," "muscle," "transcription," or "signal transduction," but also including strikingly focused categories such as "DD," "VPC," "distal tip cells," and "P lineage." To extend our work to four genomes, we generated a first-pass set of gene predictions for the newly released *C. brenneri* genome assembly. We identified 7,825 sets of strict orthologs (genes from the four species with 1:1:1:1 orthology). We also expanded our set of biological traits to include 459 microarray-based gene expression clusters encompassing 16,925 protein-coding genes, and devised tools for effectively combining multiple motif predictions through graph theory. We anticipate that the phylogenetic position of *C. brenneri* between *C. brenneri* and *C. brenneri*, along with cross-correlation of microarray data, should allow us to predict a comprehensive set of *cis*-regulatory motif candidates for *C. brenneri*.

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156. Gene regulatory network underlying patterning of gene expression and morphogenesis in the *C. elegans* vulva

Jolene S. Fernandes, Ted O. Ririe, Takao Inoue, Shahla Gharib

Establishment of spatially defined cell fate patterns is required for organogenesis. These events, in turn, depend on complex functional interconnections between regulatory genes and their targets, which should account for the morphological differences seen between species. Dissecting these gene regulatory networks entails characterizing the *trans*-acting factors that regulate gene expression and analyzing *cis*-regulatory sequences that respond to these diverse inputs. The *C. elegans* vulva invariantly consists of seven distinct cell types (vulA, vulB1, vulB2, vulC, vulD, vulE and vulF), each with its own unique gene expression profile. Diverse spatial and temporal cell fate markers, reverse genetics (RNAi), bioinformatics and the ease of manipulation at the single cell level make this a particularly attractive system for studying gene expression programs acting in organogenesis. By looking for sequence conservation in three *Caenorhabditis* species, we found vulval enhancers in over 15 genes. We then identified transcription factors that bind some of these elements through yeast one hybrid studies: for example, F53F8.1 protein, an ortholog of

human WT1, interacts with the vulval enhancer of *sqv-4* (sugarless/UGDH). Also, using Alli Mortazavi's Cistematic successfully predicted four *cis*-regulatory motifs in *zmp-1*, which were then tested by site-directed mutagenesis. Three of these motifs have demonstrable function: two positive, one negative. Meanwhile, using RNAi screens of over 500 *C. elegans* transcription factors, vulval specific factors were independently discovered by their effects on the expression of various vulval reporter gene constructs. For example, *nhr-67* (a TAILLESS ortholog) is required to inhibit the expression of *ceh-2* (EMS) and *egl-17* (FGF) in vulE and vulF cells. We find that some of these transcription factors interact with each other to specify different properties of the vulval cells. For example, both *cog-1* and *nhr-67* are mutually antagonistic and autoinhibitory in vulE and vulF cells. We also find that interconnections of these regulatory factors vary among the seven vulval cell types, which may partly account for how these cells acquire a precise pattern of fates. After integrating all the data from the above studies, we can now present a general overview of the regulatory network architecture for each of the seven vulval cell types.

157. *cis*- and *trans*-regulation of *zmp-1* expression in the *C. elegans* vulva

Ted Ririe, Jolene Fernandes

GFP reporters for approximately 32 genes are expressed in the differentiated *C. elegans* vulval cell types; each with its own spatial and temporal pattern of expression. The *C. elegans* vulva comprises seven different cell types (vulA, vulB1, vulB2, vulC, vulD, vulE and vulF). *zmp-1* (zinc metalloproteinase) is expressed in vulD and vulE starting at the late-L4 stage of development and in vulA starting in young adulthood. We are using several approaches to identify the regulatory network that controls differentiation of the vulval cell types. Vulval enhancer elements have previously been identified by analysis of the *cis*-regulatory regions of the *zmp-1* promoter. Using Ali Mortazavi's software Cistematic, four conserved motifs were found in the vulval enhancer element of *zmp-1*. Disruption of two of these sites revealed demonstrable function in driving gene expression, and disruption of a third revealed potential repressor activity. Concurrently, we have conducted RNAi screens of over 500 transcription factors to identify genes involved in the regulation of *zmp-1* expression. We have identified factors that promote *zmp-1* expression as well as factors that inhibit ectopic zmp-1 expression in unexpected cell types.

158. Linker cell migration

Mihoko Kato

Cell migration is a critical process for embryogenesis and proper development. We are studying the migration the linker cell (LC), a male-specific cell that, over the course of three larval stages, migrates through a series of turns to lead the developing gonad from the mid-body region to the cloaca in the posterior of the animal. By imaging live worms that express markers tagged with fluorescent protein, we have observed that the mechanics of migration change during the course, in particular after the mid-L4 stage as the LC approaches its destination. Using cytoplasmic YFP in the LC, we observed that although the LC retains a rounded morphology throughout most of its migration, in the mid-L4 stage it adopts a more polarized, arrowhead morphology. Timelapse confocal microscopy shows that at this late stage of its migration, the LC sends out dynamic membrane protrusions. In addition, the localization of proteins such as MIG-2—GFP (Rac), and NMY-2—GFP (non-muscle myosin) in the LC changes during this time window, suggesting a different mode of migration.

159. Behavioral/developmental metabolomics: Isolation and characterization of mating pheromones from *C. elegans*

Fatma Kaplan[*], *Jagan Srinivasan, Ramazan Ajredini*[*], *Cherian Zachariah*[*], *Hans Alborn*[*], *Aaron Dossey*[*], *Michael Stadler*[*], *Jim Rocca*[*], *Paul W. Sternberg, Art Edison*[*]

Although *C. elegans* is one of the best-studied model systems, very little is known about the chemistry that *C. elegans* uses to communicate between individuals and its environment. Several structures that secrete material to the outside have been described in nematodes, including the pharyngeal gland and the excretory/secretory system, and the intestine. However the roles of these secretions in animal communication are still not well understood. We hypothesize that *C. elegans* utilizes an extensive chemical language that allows individuals to communicate and regulates basic behaviors such as feeding, mating, and population density control. As a first bioassay to determine at what developmental stage hermaphrodites release a mating cue that is sensed by males, conditioned water of different life stages of *C. elegans* (Egg, L1, L2, L2D, L3, L4, YA and Adult) were collected. We assayed the conditioned water with Cel-him-5 males and wild-type hermaphrodites. To have a high-throughput system, we adapted the 'holding' assay as previously described (Simon and Sternberg, *PNAS*, 2002) to assay multiple animals in a single assay. This 'population holding assay estimates the time the animals spend at the spot where the conditioned water was placed versus a control spot. Our results indicate that *C. elegans* starts releasing mating cue from L4 stage till adult stage. The response to the conditioned water is sex-specific as him-five males respond more robustly than hermaphrodites. We are currently testing the sub-fractions of the young adult conditioned water for activity. Further, we are pursuing the purification of the conditioned water in two independent directions. First, a traditional forward approach of collecting large amounts of YA conditioned water is being conducted. Compounds that are released to the water are separated by various chromatographic methods, tested for biological activity, further purified, and identified by mass spectrometry and nuclear magnetic resonance (NMR). The second method is a reverse

approach by using NMR to compare worm-conditioned water of different developmental stages of worms. NMR signals that are unique to stages where mating cues are produced are identified from the mixture of compounds. These unique signals will guide HPLC purification, testing for biological activity, and identification with NMR and MS.

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160. Evidence of a mate-finding cue in the genus *Pristionchus*

Andrea Choe

The study of mate-finding behavior in simple organisms, such as *C. elegans*, has helped us to understand how specific genetic and neuron networks regulate complex survival mechanisms. There has been a growing interest in using other nematode models, given that comparative analyses assist in elucidating conserved mechanisms and reveal adaptive differences between related species. Nematodes from the genus *Pristionchus* provide one such model, sharing many similarities with *C. elegans*, yet having distinctly different modes of development and behavior. Previous studies have shown evidence of a mate-finding cue secreted by *C. elegans* hermaphrodites, which both attract and hold young adult males within close proximity (Simon and Sternberg, 2000). Here we describe evidence of a sexually dimorphic mate-finding cue in both hermaphroditic and dioecious species from the genus *Pristionchus*. To assay the mate-finding cue, we have incubated young adult hermaphrodites/females in M9 for several hours and collected the solution carrying the secretions. We then placed a small volume of the collected cue in a 5 mm diameter area on a bacterial lawn; placing an equal volume of control M9 in another 5 mm diameter area on the same lawn. Ten young adult males are then placed onto the bacterial lawn per 30-minute trial and time spent in each of the 5 mm diameter areas were recorded and compared. We have found that *Pristionchus pacificus* hermaphrodites secrete a cue that attracts and hold *P. pacificus* males within close proximity. We have also found that females from dioecious species *P. lheritieri*, *P. pauli*, and *P. uniformis* secrete a similar mate-finding cue.

161. Genes and circuits required for control of locomotory behavior during *C. elegans* male mating

Allyson Whittaker, Gary Schindelman, Shahla Gharib

To study how sensory input is coordinated with motor control we are studying genetic and neuronal pathways that control locomotory behavior during *C. elegans* male mating. Male mating behavior is arguably the most complex behavior displayed by *C. elegans* requiring integration of multi-modal sensory input to regulate specific changes in locomotory behavior. We isolated a gene, *sy682*, from a screen for mutations that disrupt mating behavior. sy682 has strong defects in the response and vulva location sub steps of mating behavior, a phenotype similar to mutations in *lov-1* and *pkd-2* the *C. elegans* orthologs of the major genes disrupted in human autosomal dominant polycystic kidney disease, but *sy682* is a new gene. We are testing whether *sy682* acts in the same, or different pathway(s) than *lov-1* and *pkd-2*. We have mapped *sy682* to a small genomic interval and are creating transgenic lines to find the gene that rescues the *sy682* phenotype. We are also interested in understanding the neuronal and genetic pathways that control changes in male tail posture during mating. We have found that bathing males in the acetylcholine esterase inhibitor aldicarb results in ventral and dorsal curling of the male tail. Ventral curling in response to aldicarb requires the M-derived muscles. Dorsal curling does not require the M-derived muscles suggesting that dorsal curling results from activation of dorsal body wall muscles and possibly by inhibition of M-derived and ventral body wall muscles. Bathing males in the nicotinic acetylcholine agonist levamisole results in both ventral and dorsal curling of the male tail and this curling requires the levamisole receptor subunit unc-29. However, mutations in unc-29 only diminish curling in response to aldicarb suggesting that other acetylcholine receptor subunits are involved in these behaviors as well. Males with mutations in *unc-29* are defective in both backing and turning behaviors. A mutation in *unc-64*, syntaxin, decreases ventral and almost completely abolishes dorsal curling in response to levamisole suggesting that levamisole acts in part through neurons for these behaviors. Some ventral but not dorsal curling in response to levamisole appears to require serotonin. Experiments are underway to determine the expression of *unc-29* in males and its site of action.

162. Transfer at *C. elegans* synapses

Anusha Narayan

Neural circuits in *C. elegans* have been studied using light and electron microscopic techniques, focal laser ablations and, more recently, calcium imaging techniques. For a clearer functional understanding of these circuits, however, some knowledge of the rules of synaptic information transfer is required. How is the dynamic range of the post-synaptic neuron set? What are the mechanisms for synaptic integration and gain control? Questions such as these can best be answered by monitoring or controlling connected pre- and post-synaptic neurons simultaneously. We chose to focus on the synapses between the AFD/ASER and AIY neurons, since the functional relevance of these neurons has been established and there is anatomical evidence for synapses between them. Channelrhodopsin-2 (chR2) is a light activated cation channel with fast kinetics (order of milliseconds[1]). We express chR2 under a neuron-specific promoter[2] in the presynaptic neuron, and use whole-cell patch-clamp recording techniques to monitor membrane voltage or currents in the postsynaptic neuron. We are first calibrating the response to light of chR2-expressing neurons. Currently, we are calibrating this light response in worms expressing chR2 in ASER. We have observed

depolarizations of 10-30 mV in response to light (450-490 nm) in current clamp, and inward currents of 5-10 pA in voltage-clamp. We have also seen evidence of spontaneous synaptic activity, in the form of discrete synaptic events (potentials or currents) with different reversal potentials (some depolarizing, others Cl-dependent). We are beginning to characterize the ASER-AIY synapse, and will then move on to the AFD-AIY synapse.

References

1. Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G. and Deisseroth, K. (2005) *Nature Neurosci.* **8**:1263-1268.
2. Nagel, G., Brauner, M., Liewald, J.F., Adeishvili, N., Bamberg, E. and Gottschalk, A. (2005) *Curr. Biol.* **15**:2279-2284.

163. Evolution of a sensory response network

Jagan Srinivasan

We are trying to understand how neural circuits evolve to mediate ethologically relevant behaviors. While model systems help elucidate how sensory information from diverse environmental conditions is represented and processed in the neural, remarkably little is known about the evolution of neural circuits, and how selective forces shape the final architecture of neural circuits and processing circuits [Dumont and Richardson (1986) *Science*]. Nematodes are an ancient phylum comprising millions of species from diverse habitats. They have molecularly diverse nervous systems that in principle can help them sense several types of environmental stimuli. To trace the evolutionary history of such a sensory repertoire, we tested three different avoidance behaviors; osmotic avoidance, response to nose touch, and volatile chemical repellence in six diverse species of free-living nematodes, a) *Caenorhabditis elegans* (N2); b) *Caenorhabditis briggsae* (AF16); c) *Caenorhabditis sp. 3* (PS1010); d) *Pristionchus pacificus* (PS312); e) *Cruznema tripartitum* (PS1351); and f) *Panagrellus redivivus* (PS2298). We found that all species tested exhibit the three avoidance behaviors. However, we also find that sensory sensitivity to the different stimuli differs among the tested nematode species. In *C. elegans*, response to these stimuli are mediated by the polymodal ASH neurons [Kaplan and Horvitz (1993) *PNAS*; Hart *et al.* (1995) *Nature*; Hilliard *et al.* (2002) *Curr. Biol.*]. We identified the pairs of putative ASH neurons in different nematode species by their anatomical positions and ablated them. Ablation of the ASH neurons in these species resulted in an inability to avoid these stimuli. By further ablation experiments, we find that there is increase or decrease in the set of sensory neurons mediating osmosensation and mechanosensation. In *P. pacificus*, osmosensation involves the ADL neuron. In *Caenorhabditis sp. 3*, mechanosensation is solely mediated by the ASH neuron as compared to three neurons in *C. elegans*. The overall conservation of ASH mediated behaviors suggests that polymodality is an ancestral feature and is evolutionarily stable, but can evolve by alterations in the level of sensitivity and the relative contributions of sensory neurons.

164. Nematodes, bacteria, and fruit flies: A tripartite model system for nematode parasitism

Elissa Hallem, Michelle Rengarajan[1], Todd Ciche[2], Paul W. Sternberg

Nematode parasitism is a worldwide health concern: over a quarter of the world's population is infected with nematode parasites, and over a hundred species of nematodes are parasites of humans. Despite the extensive morbidity and mortality caused by infection with nematode parasites, the biological mechanisms of host-parasite interactions are poorly understood. This is largely due to the lack of genetically tractable model systems for parasitism. We have demonstrated that the insect parasitic nematode *Heterorhabditis bacteriophora*, its bacterial symbiont *Photorhabdus luminescens*, and the fruit fly *Drosophila melanogaster* constitute a tripartite model for nematode parasitism and parasitic infection. We find that infective juveniles (IJs) of *H. bacteriophora*, which contain *P. luminescens* in their gut, can infect and kill *D. melanogaster* larvae. We show that infection activates a humoral immune response in *D. melanogaster* that results in the temporally dynamic expression of a subset of antimicrobial peptide (AMP) genes, and that this immune response is induced specifically by *P. luminescens*. We also investigated the cellular and molecular mechanisms underlying IJ recovery, the developmental process that occurs in parasitic nematodes upon host invasion. We find that the chemosensory neurons and signaling pathways that control dauer recovery in *C. elegans* also control IJ recovery in *H. bacteriophora*. In particular, IJ recovery is mediated by the ASJ neurons, a cGMP signaling pathway, and muscarinic acetylcholine receptors. Our results suggest conservation of these developmental processes across free-living and parasitic nematodes.

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165. Simple, inexpensive, and feature-rich single-worm tracker

Christopher J. Cronin, Eviatar I. Yemini[*], Ryan C. Lustig[*]

Past attempts at building a software and hardware solution to tracking single-worms have yielded complex and expensive results. We are developing a new open-source software solution that is simple to use, operates with a variety of inexpensive hardware, and offers a rich set of features that allow a researcher to follow and record video of a single, freely-behaving worm. This new program, titled Worm Tracker 2.0, will install with ease. It will run on a standard personal computer with any modern operating system (Windows, Macintosh, Unix variants, etc.). We expect to support most inexpensive cameras and the only major cost is incurred should be in purchasing a motorized stage and ordinary microscope, if necessary. Worm Tracker 2.0 has a rich set of features. Among them,

the program can record video of a moving worm at 30 frames/second with duration limited only by the size of the hard drive. Video can be captured intermittently in the event that a researcher wishes to observe long-term changes. The program is robust and should track worms partially obscured by food and the edges of their enclosing dish. Analysis software is included to extract over 100 features describing the worms morphology and behavior. The code is abundantly documented and supported by a growing community of developers that provide software additions, updates, bug fixes, and general troubleshooting. We hope to build a large network of labs using this new software and hardware solution to worm tracking.

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166. Large-scale neural circuit model of *C. elegans* locomotion: Mathematical modeling and molecular genetics

Jan Karbowski, Gary Schindelman, Christopher Cronin, Adeline Seah, Paul W. Sternberg

To establish the relationship between locomotory behavior and dynamics of neural circuits in the nematode *C. elegans* we combined molecular and theoretical approaches. In particular, we quantitatively analyzed the motion of *C. elegans* with defective synaptic GABA and acetylcholine transmissions, defective muscle calcium signaling, and defective muscles and cuticle structures, and compared the data with our large-scale circuit model. The major experimental findings are: (i) anterior-to-posterior gradients of undulatory frequency and especially flex both for forward and backward motion; (ii) invariance of neuromuscular wavelength; (iii) existence of some form of neuromuscular/body feedback, and, (iv) biphasic dependence of frequency on synaptic signaling. Based on the gradients of flex and frequency we hypothesize that the Central Pattern Generator (CPG) is located in the head both for forward and backward motion. Our model reveals that stretch receptor coupling in the body wall is a critical factor for generation of the neuromuscular wave and its directionality. Dynamical patterns of our circuit model are qualitatively insensitive to the details of the CPG design if stretch receptor feedback is sufficiently strong and slow. These theoretical results agree with our behavioral data, especially with (ii) and (iv), and with other pertinent published data.

167. Single worm imaging using a microfluidic platform

Oren Schaedel, Claudiu Giurumescu[1], Lap Man Lee[2], Xiquan Cui[2], Changhuei Yang[2], Paul W. Sternberg

Quantifying dynamic gene regulation can help understand the structure and the mechanism of action of gene regulatory networks. In population studies, individuals loose synchrony since they grow at different rates, resulting in a phase shift of data points. Capturing dynamic processes in single cell methods have proved efficient in bacterial cultures and in cell lines. Quantitative single animal methods are starting to emerge using confocal microscopy and time series imaging. The transparency of *C. elegans* makes it a good model for dynamic quantification of gene expression. Unlike bacterial and tissue cultured cells that stay immobilized, the natural motility of *C. elegans* raises a challenge in capturing single worm resolution images, without resolving to drug-based methods. To overcome this challenge, we are developing a microfluidic device that acts as a microenvironment. Using PDMS as a construction substrate we are able to form flow and control layers, which trap liquids but are open to gas exchange. The confined environment maintains the worm directly under the microscope, yet lets it swim around freely. Upon imaging, the ceiling of the chamber is forced down by air pressure, immobilizing the worm, and releasing it when done. Sustainability and development of the worm is achieved by a steady state flow of nematode growth buffer and *E. coli* while removing excretory products such as dauer pheromone.

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168. Automatic phenotyping and quantitative epistatic analysis

Weiwei Zhong

Genetic screens often focus on qualitative differences. For example, one screens for synthetic phenotypes to identify genetic interactions. This, however, detects only the most extreme of genetic interactions. A quantitative analysis can reveal a more complete spectrum of phenotypic changes. However, its labor-intensive nature makes it a formidable task to perform the analysis at a large scale. We are developing an automation system that employs computer vision to provide high-throughput quantitative phenotypic analysis. In its first application, the system is programmed to measure the body size of animals on a 6 cm Petri dish. We selected six genes from three independent pathways in body size regulation (TGFbeta, collagen, dosage compensation) and tested the system on single and double mutants of these genes. With accurate body length data, the systems successfully classified all genes into correct pathways. We have initiated epistatic analysis of ~100 body length genes with this system and are extending the function of the system to other phenotypic analyses.

169. Textpresso for fly: A literature search engine for researchers and curators

Hans-Michael Muller, Beverley B. Matthews[*], Susan M. Russo[*], Arun Rangarajan, William M. Gelbart[*], Paul W. Sternberg

Textpresso (www.textpresso.org) is a text-mining system for scientific literature whose capabilities go far beyond those of a simple keyword search engine. Its two major elements are a collection of the full text of scientific articles split into individual sentences, and the implementation of categories of terms for which a database of articles and individual sentences can be searched. The

categories are classes of biological concepts (e.g., gene, allele, cell, etc.) and classes that relate two objects (e.g., association, regulation, etc.) or describe one. The corpus of articles and abstracts is marked up to identify terms of these categories. A search engine enables the user to search for one or a combination of these tags and/or keywords within a sentence or document, and as the ontology allows word meaning to be queried, it is possible to formulate semantic queries. Full-text access increases recall of biological data types from 45% to 95%, and using category searches can yield a 3-fold increase in search efficiency. Originally developed for *C. elegans* literature, we have adapted Textpresso for *Drosophila* literature (www.textpresso.org/fly), by installing a corpus of 5000 full-text articles and 11000 abstracts, and by implementing fly-specific categories such as 'body part,' 'developmental stage,' 'gene (*D. melanogaster*)' and 'transposon.'

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170. WormBook: The online review of *Caenorhabditis elegans* biology

L.R. Girard, J. Mendel, D. Wang, T.J. Fiedler[1], T.W. Harris[1], F. Carvalho[1], I. Antoshechkin, M. Han[1], P.W. Sternberg, L.D. Stein[1], M. Chalfie[2]

WormBook (www.wormbook.org) is an open-access, online collection of original, peer-reviewed chapters on the biology of *Caenorhabditis elegans* and related nematodes. Since WormBook was launched in June 2005 with 12 chapters, it has grown to over 100 chapters, covering nearly every aspect of *C. elegans* research, from Cell Biology and Neurobiology to Evolution and Ecology. WormBook also serves as the text companion to WormBase, the *C. elegans* model organism database. Objects such as genes, proteins and cells are linked to the relevant pages in WormBase, providing easily accessible background information. Additionally, WormBook chapters contain links to other relevant topics in WormBook, and the in-text citations are linked to their abstracts in PubMed and full-text references, if available. Since WormBook is online, its chapters are able to contain movies and complex images that would not be possible in a print version. WormBook is designed to keep up with the rapid pace of discovery in the field of *C. elegans* research and continues to grow. WormBook represents a generic publishing infrastructure that is easily adaptable to other research communities to facilitate the dissemination of knowledge in the field.

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Publications

Antoshechkin, I. and Sternberg, P.W. (2007) *C. elegans* community resources. *Nat. Rev. Genetics* **8**(7):518-532.

Bieri, T., Blasiar, D., Ozersky, P., Antoshechkin, I., Bastiani, C., Canaran, P., Chan, J., Chen, N., Chen, W.J., Davis, P., Fiedler, T.J., Girard, L., Han, M., Harris, T.W., Kishore, R., Lee, R., McKay, S., Muller, H.M., Nakamura, C., Petcherski, A., Rangarajan, A., Rogers, A., Schindelman, G., Schwarz, E.M., Spooner, W., Tuli, M.A., Auken, K.V., Wang, D., Wang, X., Williams, G., Durbin, R., Stein, L.D., Sternberg, P.W. and Spieth, J. (2007) WormBase: New content and better access. *Nucleic Acids Res.* 35 (Database issue):D506-510.

Campbell, B.E., Nagaraj, S.H., Hu, M., Zhong, W., Sternberg, P.W., Ong, E.K., Loukas, A., Ranganathan, S., Beveridge, I., McInnes, R.L, Hutchinson, G.W. and Gasser, R.B. (2007) Gender-enriched transcripts in *Haemonchus contortus* - predicted functions and genetic interactions based on comparative analyses with *Caenorhabditis elegans*. *Int. J. Parasitol.* In press.

Ciche, T. and Sternberg, P.W. (2007) Postembryonic RNAi in *Heterorhabditis bacteriophora*: A nematode insect parasite and host for insect pathogenic symbionts. *BMC-Dev. Biol.* In press.

Feng, Z., Li, W., Ward, A., Piggott, B.J., Larkspur, E.R., Sternberg, P.W. and Xu, X.Z.S. (2006) A *C. elegans* model of nicotine-dependent behavior: Regulation by TRP-family channels. *Cell* **127**:621-633.

Fernandes, J. and Sternberg, P.W. (2007) The *tailless* ortholog *nhr-67* regulates patterning of gene expression and morphogenesis in the *C. elegans* vulva. *PLoS Genet*. **3**(4):e69.

Girard, L.R., Fiedler, T.J., Harris, T.W., Carvalho, F., Antoshechkin, I., Han, M., Sternberg, P.W., Stein, L.D. and Chalfie, M. (2007) WormBook: The online review of *Caenorhabditis elegans* biology. *Nucleic Acids Res*. **35**(Database issue):D472-475.

Hallem, E.A., Rengarajan, M., Ciche, T.A. and Sternberg, P.W. (2007) Nematodes, bacteria, and flies: A tripartite model for nematode parasitism. *Curr. Biol*. **17**(10):898-904.

Heng, X., Erickson, D., Baugh, L.R., Yaqoob, Z., Sternberg, P.W., Psaltis, D. and Yang, C. (2006) Optofluidic microscopy – a method for implementing a high-resolution optical microscope on a chip. *Lab Chip* DOI:10.1039/b604676b.

Hwang, B.J., Meruelo, A. and Sternberg, P.W. (2007) *C. elegans* EVI1 proto-oncogene, EGL-43, is necessary for Notch-mediated cell fate specification and cell invasion. *Development* **4**:669-679.

Inoue, T., Ailion, M., Poon, S., Kim, H.K., Thomas, J.H. and Sternberg, P.W. (2007) Genetic analysis of dauer formation in *Caenorhabditis briggsae*. *Genetics*. In press.

Karbowski, J., Schindelman, G., Cronin, C.J., Seah, A. and Sternberg, P.W. (2007) Systems level circuit model of *C. elegans* undulatory locomotion: Mathematical modeling and molecular genetics. *J. Comput. Neurosci*. In press.

Kee, Y., Hwang, B.J., Sternberg, P.W. and Bronner-Fraser, M. (2007) Evolutionary conservation of cell migration genes: From nematode neurons to vertebrate neural crest. *Genes & Dev*. **21**:391-396.

Modzelewska, K., Elgort, M.G., Huang, J., Jongeward, G., Lauritzen, A., Yoon, C.H., Sternberg, P.W. and Moghal, N. (2007) An activating mutation in *sos-1* identifies its Dbl domain as a critical inhibitor of the EGFR pathway during *C. elegans* vulval development. *Mol. Cell Biol*. **27**(10):3695-3707.

Van Buskirk, C. and Sternberg, P.W. (2007) EGF signaling induces behavioral quiescence in *C. elegans*. *Nature Neurosci*. In press.

Zhong, W. and Sternberg, P.W. (2007) Automated data integration for developmental biology research. *Development* **134**:3227-3238.

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Visiting Associate: Eric Mjolsness
Visiting Associate: Sandra Sharp
Graduate Students: Katherine Fisher, Gilberto Hernandez Jr., Anthony Kirilusha, Steven Kuntz, Ali Mortazavi, Tracy Teal, Nicole Tetreault
Research and Laboratory Staff: Tristan DeBuysscher, Brandon King, Kenneth McCue, Dolores Page, Diane Trout, Brian Williams

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Summary: In the Wold group we are interested in the composition, evolution and function of gene regulatory networks. We often use muscle development, degeneration, and regeneration as a favored model system. We are especially interested in networks that govern how cell fates and states are specified, executed, and maintained. Our approaches to these problems make heavy use of genome-wide and proteome-wide experimental assays coupled with informatic and computational tools designed to extract connectivity relationships and evolutionary relatedness. Evolution of networks is studied in collaboration with Paul Sternberg and his group to compare our mammalian networks with orthologous networks in worms and with John Allman and his group for comparative studies of brain networks in primates and rodents.

A longstanding challenge for us is to understand the regulatory events that drive the progression from multipotential precursor cells or adult stem cells to determined unipotential progenitors and then to fully differentiated cells. We currently study these cell states and transitions using a new set of functional genomics tools based on new ultra high throughput DNA sequencing methods coupled with comparative genomics. In mouse and other vertebrates, this lineage arises from paraxial mesoderm to produce muscle (also bone, skin and fat, among other derivatives). Skeletal myogenesis is governed by both positive- and negative-acting regulatory factors. The MyoD family of four closely related, positive-acting transcription factors are key. Upon transfection, each can trigger nonmuscle recipient cells enter, and often to execute, the myogenic differentiation program. Our working model emphasizes that MyoD family factors are able to do this because they are highly connected and cross-talk within their group and with other collaborating factors (like MEF2 factors) and other regulators that now include microRNAs. This frames the problem in network terms. It is also clear that negative regulators of skeletal myogenesis are probably just as important for regulating the out come as are the positive regulators. Multiple negative regulators of skeletal muscle are expressed in multipotential mesodermal precursors and in proliferating muscle precursors (myoblasts). It is generally believed that some of these are important for specifying and/or maintaining precursor cells in an undifferentiated state, though exactly how the system works is unknown. This focuses attention on defining the *in vivo* target repertoire for the repressors, and learning their relationship with the positive-acting regulators.

Because we needed to learn the genome-wide input and output maps for multiple activating and repressing regulators, we were motivated to develop ChIPSeq and RNASeq techniques for making global *in vivo* protein:DNA interaction maps and RNA expression profiles (Johnson *et al*., 2007 and entries of Williams and Mortazavi below). By mapping global *in vivo* factor occupancy maps for key regulators and learning how they change from one cell state to another, we aim to address some longstanding puzzles: What distinguishes binding motifs in the DNA that are actively bound by their cognate regulators *in vivo* from other motif instances that are not similarly bound? How does the occupancy connectivity map predict the transcriptional output of target genes? What is the target promoter repertoire for a given bound regulator on the chromosome? Work by Brian Williams, Ali Mortazavi, Tony Kirilusha and Katherine Fisher all contribute to an effort that aims to map all target sites of – ultimately - all transcription factors significantly expressed in muscle precursors and myocytes.

The MUSSA/MUSSAGL informatics tools described below (work of Tristan DeBuysscher, Diane Trout and Brandon King) have been used to find candidate conserved regulatory elements, and these are, in turn, being subjected to functional assays via lentiviral-mediated transgenesis. We are using a similar approach to analyze data from multiple species of worms related, in differing degrees, to *C. elegans*. This is part of an ongoing partnership with the Sternberg lab that involves several joint projects (see Steven Kuntz and Ali Mortazavi entries below and in the Sternberg Section). In addition to clarifying how many and which worm genomes give us the most leverage for identifying functionally important noncoding elements in the genome, we seek insights into the evolution ancient gene networks that drive myogenesis and cell cycle across the large phylogenetic distances separating vertebrates, worms, flies and for cell cycle, even plants and yeasts.

To define protein:protein complexes in the network more comprehensively, we developed a collaborative effort with the Deshaies lab here and the John Yates lab at Scripps to modify and apply MudPIT mass spectrometry, coupled with dual affinity epitope-tagging, to characterize multiprotein complexes (Graumann *et al.,* 2004). Among other things, this enabled postdoctoral fellow Libera Berghella to identify a new repressor that acts on myogenin, the MyoD family member most directly responsible for execution of differentiation.

Tracy Teal's study of biofilm development is a collaboration with Diane Newman in the Division of Geology and Planetary Science. It has nothing to do with myogenesis, but it does concern a different kind of development. Its goal is to identify, visualize, and

ultimately understand the multiple different metabolic cell states that comprise a biofilm at different stages of its development and under differing environmental stimuli. The degree to which principles and regulatory strategies used by metazoans during development are or are not employed by bacteria in creating biofilm structures is being probed by marking bacteria with multiple GFP derivatives driven by genes that will mark functional domains (aerobic vs. anaerobic, for example). To do this, Tracy developed a new and elegant way to characterize biofilm morphogenesis quantitatively. She then used it to establish that the widely held view of mature biofilms having cores composed of dead cells is incorrect, and that these cores are instead in a distinct metabolic and growth state. This quiescent but nevertheless live and active state may help to explain extreme biofilm robustness under diverse environmental stimuli, including antibiotic treatment (Teal *et al*., 2006).

Reference

Graumann, G. Dunipace, L.A. Seol, J.H., McDonald, W.H., Yates Jr. III, J.R., Wold, B.J. and Deshaies, R.J. (2004) *Mol. Cell Proteo*. **3**:226-237.

171. ChIPSeq: Genome-wide mapping of transcription factor:DNA interactions

Ali Mortazavi, Ken McCue, David Johnson[*]*, Rick Myers*[*]

In vivo protein-DNA interactions connect each transcription factor with its direct targets to form a gene network scaffold. Although much is known about transcription factor binding and action at specific genes, far less is known about the composition and function of entire factor:DNA interactomes, especially for organisms with large genomes. Now that human, mouse, and other large genomes have been sequenced, it is possible in principle to measure how any transcription factor is deployed across the entire genome for a given cell type and physiological condition. Direct physical interactions between transcription factors or cofactors and the chromosome can be detected by chromatin immunoprecipitation. In ChIP experiments, an immune reagent specific for a DNA-binding factor is used to enrich target DNA sites to which the factor was bound in the living cell. The enriched DNA sites are then identified and quantified.

For the gigabase-size genomes of vertebrates, it has been difficult to make ChIP measurements that combine high accuracy, whole-genome completeness, and high binding site resolution. These data quality and depth issues dictate whether primary gene network structure can be inferred with reasonable certainty and comprehensiveness, and how effectively the data can be used to discover binding site motifs by computational methods. For these purposes, statistical robustness, sampling depth across the genome, absolute signal and signal-to-noise ratio must be good enough to detect nearly all *in vivo* binding locations for a regulator with minimal inclusion of false positives. A further challenge is to map factor-binding sites with high positional resolution. In addition to making computational discovery of binding motifs feasible, this dictates the quality of regulatory site annotation relative to other gene anatomy landmarks such as transcription start sites, enhancers, introns and exons, and conserved noncoding features. We also think that if high quality protein:DNA interactome measurements can be performed routinely, we will be able to do detailed studies of interactome dynamics in response to specific signaling stimuli or genetic mutations. To address these issues, we turned to ultra high-throughput DNA sequencing with a Solexa/Illumina instrument.

We used the NRSF repressor to work out the ChIPSeq method because we had previously generated a detailed genome-wide computational model for its target sites along with a large set of individually measured, validated true positive and true negative sites (Mortazavi *et al*., 2006). Critical to the project was the monoclonal immune reagent for NRSF derived by the Anderson lab at Caltech and generously supplied to us. The resulting sequence census method was used to map *in vivo* binding of the neuronal restrictive silencing factor NRSF/REST, to 1,946 locations in the human genome. The data display sharp resolution of binding position (+/-50 bp), which facilitated motif finding and allowed us to identify new, noncanonical NRSF binding motifs. These ChIPSeq data also have high sensitivity and specificity (ROC area $\geq$ 0.96) and statistical confidence ($P< 10^{-4}$), properties that are important for inferring new candidate interactions. New targets of NRSF found in this was included key transcription factors in the gene network that regulates pancreatic islet cell development.

Current informatics work includes development of more advanced methods for detecting ChIPSeq signals and for subdividing compound regions of factor occupancy into their individual contributing sites. Current experimental work involves extension to different classes of factors and measurement of co-occupancy by multifactor complexes.

172. RNASeq: Transcriptome profiling by direct DNA sequencing

Ali Mortazavi, Brian Williams

Transcriptome profiling has been made possible by microarray methods. These measurements are very useful to us as a way of measuring the global output of gene networks. The ideal profile is one that is very high signal to noise and – ideally – assays the entire genome to permit discovery of previously unrecognized genes. To bypass some limitations of microarray systems, we worked out an RNASeq method for mapping and measuring amounts of RNA. We used the "sequencing by synthesis" instrument from Solexa/Illumina. The data produced are a large collection (generally 1-10 million reads per profile) of short (~30NT long) sequence reads that are computationally mapped back to their sites of origin in the genome. Informatics challenges involve mapping splice junctions, new gene discovery, and allocating sequence reads among paralogous genes in the genome. Experimental challenges include improving uniformity of cDNA copying and for preparing RNAs from for RNASeq form very small numbers of cells or from previously fixed

tissues. We are also developing a series of standards in order to better measure the sensitivity of these methods and of corresponding microarrays. The first applications are to brain RNA samples (see Tetreault and Williams *et al*., below) and to myogenic network mapping (see Williams, Kirilusha *et al*., below).

173. Skeletal muscle transcriptional regulatory network using ultra high throughput sequence counting methods

Brian Williams, Anthony Kirilusha, Katherine Fisher, Gordon Kwan, Sandy Sharp, Ali Mortazavi

Progression from undifferentiated precursor cells to differentiated cells with identifiable functional traits is regulated primarily at the level of gene transcription. Genome-scale experimental approaches allow us to assay all possible molecules that emerge from the genome during the differentiation step, with the objective of grouping them into logic circuits that explain how the cell regulates and executes differentiation. A first approach is to correlate changes in the genome-wide transcription factor site occupancy map that occur during the differentiation transition, with changes in RNA output from the genome. Transcription factor-site occupancy maps are being developed by immunoprecipitating chromatin preparations from the C2C12 skeletal muscle cell line using antibodies for transcription factors known to be key regulators of muscle differentiation along with more general regulators of transcription activation and repression. Shared analysis work in the project is to integrate occupancy maps and transcription output for factors that include MyoD, myogenin, E47, Mef2, NRSF, SRF and others.

174. A new repressor mediates transcriptional down-regulation of myogenin during innervation

Libera Berghella, Shirley Pease

Myogenin, one of the four-member MyoD family of bHLH myogenic transcription regulators, is a crucial regulator of myogenesis that is down-regulated at the RNA level during muscle fiber maturation as a consequence of innervation. Comparative genomic analysis of sequences flanking myogenin using an early version of MUSSA (see entry from Tristan De Buysscher below), highlighted a highly conserved 17bp element (myogHCE). We asked what its function is, first by using lentiviral-mediated transgenesis. This showed that it mediates and is essential for postnatal repression of myogenin in transgenic animals. Subsequent mass spec analysis of proteins enriched by binding to this motif identified a candidate transacting factor from adult muscle that could be responsible for this regulatory action. It binds myogHCE *in vitro* and occupies the locus in adult skeletal muscle. Altering its levels experimentally shows that it can repress differentiation in C2C12 moblasts in the gain of function mode, and that it can also regulate multiple effects of innervation in adult muscle. It binds the myogHCE motif as part of a complex that includes the homeodomain protein Pbx. Genome-wide analysis identified a family of related conserved elements, one of which is located 35 Kb upstream of MyoD in mouse and also interacts with MSY-3 *in vivo*. Taken together, these results suggest MSY-3 is a new component of the muscle regulatory network, critical for proper innervation-dependent activity during development.

175. Automation of genome-wide and cross-genome *cis*-regulatory element identification and assessment using Cistematic

Ali Mortazavi, Sarah Aerni[*]

We are continuing development of an extensible computational framework called Cistematic, which is designed to automate discovery and refinement of candidate *cis*-regulatory motifs. It further performs genome-wide phylogenetic mapping for the motif or motif combinations of interest. The objective is to stratify motif occurrences by applying user-specified criteria for phylogenetic conservation and/or site position relative to adjacent coding sequences or other genome features. Motif occurrences that are most conserved are identified using the *cis*Matcher algorithm, which assesses conservation without need of pre-computed multiple-sequence alignments. Resulting sets of conserved motif occurrences, together with identities of the adjacent genes, comprise a "*cis*-motif cohort." A Gene Ontology enrichment module in Cistematic can then be used to test whether a *cis*-motif cohort is significantly enriched or depleted in members with specific GO functions. This analysis path is especially straightforward and applicable for relatively large-binding motifs such as those typical of many zinc-finger transcription factors.

All data is stored transparently in relational form using the cross-platform Sqlite package. Cistematic provides several layers of abstraction, which allow users of varying levels of sophistication to customize their uses of Cistematic. Thus, while most users will simply supply locus identifiers, and thresholds to the experiment classes and receive standard analysis results, some users may wish to use and extend lower-level objects that handle Motifs, Programs, Genomes, and Homology/Annotation mapping.

We are further expanding Cistematic by adding native implementations of the greedy, Gibbs sampler, and Expectation Maximization motif finding algorithms that form the basis of most *in silico* motif finders used in the field in order to make them available "out of the box." Cistematic currently runs on Mac OS X, Linux, and Solaris, and has a prototype web front-end for users wishing to run Cistematic experiment objects without writing Python scripts.

Application of Cistematic to the problem of global motif finding and comparison in *C. elegans* and related worm genomes is currently in progress in collaboration with Erich Schwartz in Paul Sternberg's group.

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176. Inferring the structure of the yeast cell cycle transcription network by neural network modeling

Christopher Hart, Eric Mjolsness

A prominent network of kinases (Cdks), together with a coupled system of regulated proteolysis, governs progression of the yeast cell cycle. A major downstream output of this signaling system is transcriptional regulation of a large set of genes, some of which are known to play important roles in further regulating and executing the phases of the yeast cell cycle. Between 300 and 1000 yeast gene RNAs are expressed in a cell cycle-dependent manner. This wide range in the number of genes designated as cycling depends on a combination of experimental specifics, such as the method of phase synchronization, the analytical methods, and criteria for defining cell cycle regulation, but a core of ~200 are common to virtually all studies, and the major kinetic patterns correspond roughly to the phases of the cell cycle. In this project, I used artificial neural networks (ANNs) to integrate results from genome-wide time-resolved RNA expression data from microarrays with large-scale measurements of genome-wide protein:DNA interactions from ChIP-on-chip experiments. The motivation for this method is that this architecture of neural network is very simple, yet captures and capitalizes on several properties of gene networks that are not used by other established methods of analysis. By mining the network weights matrix, 10 of 12 previously known regulatory connections that are associated with the cell cycle by traditional molecular genetics and biochemistry emerged as top connections in the ANN. In addition, several novel connections scored highly by the ANN. These new model-based connections provide a basis for hypotheses about additional regulators acting in the cell cycle. With each of these regulatory relationships we also capture the cell cycle phase in which these regulatory associations are likely to be pertinent.

177. Genome-wide comparative analysis of the NRSF/REST target gene network

Ali Mortazavi

We are investigating the role of a major transcriptional repressor in the evolution of the corresponding gene regulatory network (GRN). We are using a combination of computational prediction and direct experimentation to define the genome-wide set of direct targets of the well-known Neuron-Restrictive Silencer Factor (NRSF/REST). This repressor was originally identified as a global repressor of neuronal genes in non-neuronal tissues in work in the Anderson lab at Caltech in the late 80's. It has more recently been shown to repress neuronal genes in stem cells prior to their differentiation. Roles for it have also been suggested in pancreatic development, cardiac lineages, and lymphocyte lineages, although it is not clear whether these roles differ from repression of neuronal genes in non-neuronal tissues during embryogenesis. NRSF is an ideal model factor for defining a regulatory network from the trans-factor point of view, in part because it has a long (21 bp) and relatively well-defined binding motif. Basic network architecture questions include: What are all the *in vivo* targets of NRSF? In what cells are they occupied by the factor and with what consequences? How has this network evolved since its beginnings in the vertebrate lineage?

Our first approach to these questions used Cistematic (see Mortazavi and Aerni above) across human, mouse and dog genomes to leverage evolutionary conservation to refine the motif model and to locate instances and their adjacent genes. The major experimental tests of sites identified computationally, along with sites of the same size and similar base composition that are predicted not to bind NRSF, are ChIP assays (chromatin immunoprecipitation). Initially ~100 predicted sites from the group of ~700 were assayed by Q-PCR. These allowed us to evaluate the predictive success of our model of the human NRSF target repertoire in Jurkat cells (Mortazavi *et al.*, 2006). We are finding similar site occupancy patterns in mouse muscle cells. The composition of the computational target gene set was also interesting because it includes multiple microRNAs and regulators of neuron specific splicing. Among these are microRNAs predicted by sequence to be plausible regulators of NRSF and its Co-Repressor, CoREST, suggesting a dynamic feedback. The perfect conservation of the entire zinc-finger set that comprises the NRSF DNA-binding domain throughout all sequenced vertebrates is striking. Coupled with the absence of any identifiable NRSF transcription factor in all sequenced invertebrates, suggests the notion that the emergence of NRSF in the vertebrates may have been needed to permit "reuse" of neuronal genes in non-neuronal, vertebrate-specific regulatory networks.

To further test and refine the computationally-derived model of the NRSF interactome, we worked with Dave Johnson in the Myers lab to measure the entire global "NRSFome" experimentally by using microarray and very high throughput sample sequencing (e.g., Solexa) to make ChIP measurements (see first entry above). We are now using ChIPSeq to map NRSF interactomes in mouse, horse, and dog genomes, for comparison with the human version. The aims are to determine which target genes, and which detectably conserved binding sites, are used in all interactomes; how many new NRSF/target relationships emerge in each genome, and how many have disappeared. This work will also investigate how the NRSF interactome map changes from one cell type to another within the same specie. In the first NRSF ChIPSEq work, performed in human T cells, we found that NRSF binds to a family of previously unappreciated half-site motifs as well as to its canonical full site (Johnson *et al*., 2007). This suggested a working hypothesis for the evolution of strong full-length sites from a pool of less optimal half-sites separated by flexible spacing. Patterns of site evolution and use across the four species in the current work should begin to test this idea.

178. *cis*-Regulatory analysis of a Hox cluster using multiple nematode sequences

Steven Gregory Kuntz

Identifying animal *cis*-regulatory modules has often required laborious *in vivo* promoter mutagenesis that ignores genomic conservation. Though some studies in *Caenorhabditis elegans* have used pairwise comparisons to highlight putative modules by conservation, in theory using more genomes at appropriate evolutionary distances should allow greater predictive power [Eddy *et al.* (2005) *PLoS Biol.* **3**, e10; Stone *et al.* (2005) *Annu. Rev. Genomics Hum. Genet.* **6**:143-164]. Technical and biological uncertainties make it unclear how well this will actually work for any given clade. To accelerate studies of transcriptional regulation, it is critical to determine in nematodes how much more efficiently phylogenetic footprinting with N-way comparative genomics can identify *cis*-regulatory elements than uninformed promoter dissection. We are testing the ability of five *Caenorhabditis* genomes (*C. elegans*, *C. briggsae*, *C. brenneri*, *C. remanei*, and *C.* sp. 3 PS1010) to identify candidate non-coding regulatory modules. The *ceh-13/lin-39* Hox clusters of these nematodes were computationally scanned for ungapped sequence similarity with the MUSSA algorithm, an N-way generalization of Family Relations. The entire non-coding sequence was divided into eleven highly conserved regions — each consisting of a conserved site and flanking sequence — and ten less conserved regions. Following *in vivo* transgenic tests, we calibrated the analysis with an empirical threshold to separate the highly conserved from the less conserved regions. All nine domains above the empirical calibration threshold directed regulatory activity, giving 100% specificity, and only three below-threshold regions directed regulatory activity, yielding a 75% recovery rate. This suggests that nematode *cis*-regulatory elements are strongly enriched in or near sites of ungapped sequence conservation. Surprisingly, some of these elements have similar sequences that appear to be highly conserved in the Hox clusters of several different vertebrates. We are currently testing these sites and searching for examples in other phyla to determine whether they may have a common origin.

179. Gene expression profiling of primate brain regions and cells that mediate social interaction

Brian Williams, Nicole Tetreault, Virginie Goubert, John Allman

Von Economo neurons (VENs), are a recently evolved population of specialized neurons found in layer 5 of the human cortex. They are believed to be involved in the perception of social emotions, and intuitive assessment of complex, uncertain circumstances. They may be dysfunctional in patients exhibiting behaviors characteristic of the autism spectrum. These relationships have been deduced largely from anatomical and pathological evidence, but little is known about the molecular regulation of VEN formation and function. In this collaboration with the Allman lab, we are developing a transcriptional profile of mRNAs expressed in brain domains strongly associated with regulation of social behavior, and then within these areas, profiles of the VENs themselves. Deep profiling by RNASeq aims first to discover a defining set of molecular markers (and combinations of them) for VENs and for other cells involved in mediating aspects of social behavior. We will also seek characteristic genes and gene clusters to suggest hypotheses concerning the development and evolution of the neuronal circuitry that includes VENs. At the cellular level, one goal is to identify all ion channels and expressed in VENs to inform functional modeling efforts, and another is to seek clues to their unusual morphology. Initial studies focus on anterior cingulated cortex and frontoinsular corte, plus nearby regions that lack VENs and mediate different functions, and later experiments pursue laser-dissected VEN cell samples. Since the VENs are only a small fraction of all the cells located in their native subregions of the brain, we are using an immunological labeling and *in situ* hybridization to test and validate candidate genes emerging from the profiling effort. To begin, we have produced labeled RNA probes for three receptors for reward type signals (dopamine receptor D3, serotonin receptor 2b, and the vasopression receptor 1A) shown by prior research to be expressed in VENs, and are working out a method for *in situ* hybridization to identify their expression in both human and mouse brain sections. As candidates are validated, we hope to be able to expand the scope of this project to trace evolution of VENs and related cells, including possible antecedents in mice where genetic manipulations would be possible.

180. Microfluidic chaotic mixing devices improve signal to noise ratio in microarray experiments

Jian Liu, Brian Williams, Richele Gwirtz, Stephen Quake

In conventional microarray experiments, hybridization of labeled target DNA molecules to sequence-specific probes relies on diffusion in a static hybridization solution volume. During the time interval for a conventional microarray experiment, a target molecule can be expected to traverse a distance of about 1-3 mm. The dimensions of a typical microarray are >10 mm, therefore, a large portion of the available labeled target does not encounter its cognate sequence probe for specific hybridization. We have attempted to improve the performance of microarray hybridization using a microfluidic chaotic mixing chamber to perform dynamic hybridization. We fabricated a two-layer polydimethylsilicone (PDMS) microfluidic mixing/ hybridization chamber and sealed it to a spotted microarray slide. The fluidic layer of the device contains two symmetric hybridization chambers (6.0 mm x 6.5 mm X 65 microns). They are connected to each other by bridge channels, whose ceiling contains a series of indentations in a herring-bone pattern, which produces chaotic mixing of the hybridization solution. Four input/output through-holes with corresponding micromechanical valves are used for loading sample solutions or disposing waste buffers. The valves are actuated to form closed chambers during the circulation of the fluid. Additionally, in the control layer two sets of

peristaltic pumps are integrated to move the fluid between the hybridization chambers.

Test hybridizations were performed on spotted microarrays containing 4321 gene-sequence-specific 70 mer oligonucleotide probes, with an additional 65 randomized sequence 70 mer probes as negative controls. Both static and dynamic hybridizations were performed for a 2-hr time interval, and hybridization intensities for positive probes and negative controls were compared. Receiver-operating characteristic (ROC) curves for the two experiments indicate a much greater separation in the distribution of positive probe intensities compared to negative control intensities for the dynamic hybridization experiment than for the static experiment (area under the ROC curve for dynamic hybridization = 0.92; area for static hybridization = 0.73). Experiments are in progress to test the performance of these methods at longer hybridization intervals. The dynamic mixing method has the potential to increase the detection sensitivity for conventional spotted microarrays at a relatively low cost. It is compatible with commercially available microarray slides, and offers the possibility of integration into large-scale nucleic acid sample processing operations.

181. *In vitro* degeneration system for skeletal muscle

Brian A. Williams, Gilberto Hernandez, Jr.

Skeletal muscle is one of the most noticeably compromised tissues during normal human aging, and undergoes active degeneration (cachectic wasting) during neoplastic disease progression. We are attempting to understand the molecular regulation of this process by modeling skeletal muscle degeneration *in vitro* using the C2C12 skeletal muscle cell line. Our previous results indicate that members of the E3 ubiquitin ligase family, which have been shown to be upregulated during degeneration of skeletal muscle tissue, are substantially expressed during the normal differentiation of C2C12 cells when cultured under atmospheric oxygen conditions. Previously published work has indicated that generators of reactive oxygen species (ROS) such as hydrogen peroxide can cause upregulation of the E3 ubiquitin ligases. We are manipulating culture conditions in order to cause inducible expression of the E3 ligases, with the goal of identifying specific transcriptional regulatory effects on a genome-wide scale. We are also developing more sensitive and reliable quantitative real time RT-PCR assays for a small battery of genes known to play a role in degeneration and stress response. cDNAs for this battery have been cloned and expressed as RNA molecules, for use as "spike-in" controls in quantitative RT-PCR experiments.

182. Spatiometabolic stratification of *Shewanella oneidensis* biofilms

Tracy Teal, Dianne Newman

Biofilms, or surface-attached microbial communities, are both ubiquitous and resilient in the environment. It is now recognized that many bacteria in the environment grow as surface-attached microbial biofilms, and it has been suggested that this biofilm lifestyle may account for the remarkable persistence of bacterial populations in the face of changing environmental conditions. Biofilms are composed of many hundreds to millions of cells, each of which experiences its own microenvironment due to strong chemical gradients that are established by metabolism and diffusion. Biofilms are therefore heterogeneous and spatially stratified, so that activity levels and biochemical processes occur differentially according to the location of a cell in the biofilm and the biofilm structure's biomass.

Although much is now known about how biofilms form, develop and detach, very little is understood about how these events are related to metabolism and its dynamics. It is commonly thought that large sub-populations of cells within biofilms are not actively producing proteins or generating energy, and are therefore dead. Early results in this project led us to hypothesize that within the growth-inactive domains of biofilms, significant populations of living cells persist and that they retain the capacity to dynamically regulate their metabolism. To test this, we employed unstable fluorescent reporters to measure growth activity and protein synthesis *in vivo* over the course of biofilm development and created a quantitative routine to compare domains of activity among independently grown biofilms. We found that *S. oneidensis* biofilm structures do indeed reproducibly stratify with respect to growth activity and metabolism as a function of their size. Moreover, within domains of growth-inactive cells, genes that are typically upregulated under anaerobic conditions are expressed well after growth has ceased. These findings show that, far from being dead, the majority of cells in mature *S. oneidensis* biofilms have actively turned on metabolic programs appropriate to their local microenvironment and developmental stage (Teal *et al.*, 2006). This raises a new set of questions concerning the relationship of these cells in a biofilm to the stationary phase of cells growing in planktonic culture; the full range of metabolic activities performed by these cells; and the nature of signaling systems that lead to defining this state.

183. Transcriptional and post-transcriptional regulation of skeletal muscle atrophy

Gilberto Hernandez, Jr.

My goal is to identify and then learn the function of components of the gene network controlling muscle atrophy genes. A major contributor to the profound stereotypic proteolysis seen in skeletal muscle atrophy is the ubiquitin proteosome pathway, and two key E3 ubiquitin ligases mediate much of this effect. They are Muscle Atrophy F-box (MAFbx) and Muscle RING Finger-1(MuRF-1). Current understanding of the signaling cascade(s) regulating muscle mass have already benefited from the use of MAFbx and MuRF-1 as primary regulatory targets and molecular effectors of atrophy. However, the explicit transcriptional regulatory network controlling MAFbx and MuRf-1 remains largely unknown. By working from functional *cis*-regulatory elements, identified partly by their conservation through evolution and partly by experiment, we want to learn how these genes, and others with similar expression dynamics *in vivo*, are regulated. In addition, we hope to use these

cis-acting regulatory regions, and sequence motifs within them, to identify other genes that share them and are part of the atrophy response gene network.

Using comparative genomic sequence analysis, I made a catalog of non-coding, putative *cis*-regulatory elements for both Mafbx and Murf-1 genes and established an *in vitro* atrophy assay, which recapitulates the initial suppression and activation of these genes. Sequence similarity analysis and a focus on shared motifs within 3'UTR regions has led a focus on specific elements as predicted target sites for transcription factors binding or for microRNA interaction.

Reference

Hart, C.E., Sharenbroich, L., Bornstein, B.J., Trout, D., King, B., Mjolsness, E. and Wold, B.J. (2005) *Nucl. Acids Res.* **33**(8):2580-2594.

184. BioHub

Brandon King, Joe Roden

The goal of the BioHub database project is two-fold. The first is to provide our biologists with a tool that allows them to ask questions of very different kinds of large-scale biological data which are tied together based on their spatial relationship to a gene or DNA sequence feature in one or more genomes. The computational goal is to make a rich API (Application Programming Interface) to allow computer scientists to easily write custom large-scale analysis programs, which can then be turned into web application or other GUI to allow for easy to use large-scale analysis.

In it's current form, BioHub is a spatial annotation PostgreSQL database with a Python API for writing applications. It works by registering sequences (annotations of sequences) in the BioHub core database. Upon registering an annotation at a location on a genome, the user or program receives an SID (Sequence ID) that can later be used as a handle to the 'Registered Sequence' when using the BioHub API. An SID will always be the same for the exact same location on a genome. This means that if two different programs or people register a sequence with the exact same location, both will be given the exact same SID. This feature is important because it allows for connection of a wide variety of biological data to associated by simply having the same location on the genome. For example, if one were to register a sequence they found in a publication as a 'conserved regulatory motif' and then later a motif finding program finds the exact same motif, they will both have the exact same SID. But they will also have two descriptions and users attached to the SID, as well. One saying "found in paper x..." and the other "discovered by motif finding program y." By simply registering the two sequences, the published motif has now been connected to all sites in BioHub, and in current work the hub is expanding to allow the next obvious query to recover all expression data associated with this custom set of instances. The user has the capacity, through BioHub, to specify and collect, via SIDs, only those genes associated with motif instances that have a particular positional relationship to your gene models. BioHub was used to design a custom gene chip that discriminates hundreds of related zinc-finger transcription factors in the human genome. These are not well represented with non-crossreacting probes in current commercial array collections.

185. Comparative genome analysis over more than three genomes using MUSSA and MUSSAGL

Diane Trout, Tristan De Buysscher, Brandon King

Comparative genome analysis, as a routine lab tool for cell and molecular biologists, is becoming increasingly important as the number of fully sequenced genomes increases. In general, the more genomes included in an integrated comparative analysis, the better the resolution of functional elements conserved due to selection, versus sequences that are the same by chance. Great evolutionary pressure in such a comparison can also be exerted by identifying the very rare elements that are recognizably similar over a single very long distance, such as human to fish. However, we find that this approach, as would be expected, is very severe and has the effect of eliminating many known functional enhancers and promoters that we want to be able to detect. The availability of many mammalian genome sequences means that we can get evolutionary leverage by integrating over larger numbers of genomes each of which is individually not so distant from the reference mouse or human genome. Once these basic comparisons are made we want to relate domains of sequence conservation to each other, ask if they have shared internal elements, and map additional features such as small transcription factor-binding motifs, regulatory RNA interaction motifs, etc. MUSSA (multiple sequence similarity assignment software) is an interactive viewer that was designed to do analysis of this kind, at the level ~100 kb regions for N genomes, rather than for two or three. It uses a transitivity-filtering algorithm to integrate sequence similarities over the entire collection of genomes being compared. The underlying sequence similarity mapping was done with a classic sliding window method, implemented as it was for the earlier FR project with the Eric Davidson lab. MUSSA interactivity features permit inspection of the analysis at varying levels of resolution, recovery of specific sequence regions for further external analysis, and user-driven integration of conserved sequence domains with maps of sequence motifs such as transcription factor-binding sites or gene model annotations. MUSSA analysis has been done on multiple worm genome sequences with Erich Schwarz and Paul Sternberg and Steven Kuntz, and on several genes of interest in the myogenic regulatory network from varying numbers of mammalian genomes. These comparisons highlight both known and new candidate sequence domains and candidate factor-binding motifs within those domains. Experimental tests for regulatory function in transgenic assays are underway and a majority of noncoding conserved elements from the lin39-ceh13 Hox locus are proving to be active. MUSSA is available from the Wold website.

186. A PCA-based way to mine large microarray datasets

Joe Roden, Chris Hart, Brandon King, Diane Trout

In many instances where large-scale microarray analysis is part of a project, the biologically important goal is to identify relatively small sets of genes that share coherent expression across only some conditions, rather than all or most conditions, which is what traditional clustering algorithms find. The PCA (principle components analysis)-based tool developed in this project performs a complementary kind of datamining that helps one identify groups of genes that are highly upregulated and/or down-regulated similarly, across only a subset of conditions. Equally important is the need to learn which conditions are the decisive ones in forming such gene sets of interest, and how they relate to diverse conditional covariates, such as disease diagnosis or prognosis. This is a nontraditional use of PCA, and the software tools for doing it will be accessible from the group website. The method is described in a recent paper (Roden *et al*., 2006) and the software is openly available at the Wold group website.

Publications

Hart C.E., Mjolsness, E. and Wold, B.J. (2006) Connectivity in the yeast cell cycle transcription network: Inferences from neural networks. *PLoS Computation* **3**.

Johnson, D., Mortazavi, A., Myers, R. and Wold, B. High-resolution genome-wide mapping of *in vivo* protein DNA interactions. *Science* May 31 2007; 10.1126/science.1141319.

Liu, J., Williams, B.A., Gwirtz, R.M., Wold, B.J. and Quake, S. (2006) Enhanced signals and fast nucleic acid hybridization by microfluidic chaotic mixing. *Angew Chem. Int. Ed. Engl*. **45**:3618-3623.

Mortazavi, A., Thompson, E.C., Garcia, S.T., Myers, R.M. and Wold, B. (2006) Comparative genomics modeling of the NRSF/REST repressor network: From single conserved sites to genome-wide repertoire. *Genome Res.* **16**:1208-1221.

Roden, J.C., King, B.W., Trout, D., Mortazavi, A., Wold, B.J. and Hart, C.E. (2006) Mining gene expression data by interpreting principal components. *BMC Bioinformatics* **7**:194.

Teal, T.K., Lies, D.P., Wold, B.J. and Newman, D.K. (2006) Spatiometabolic stratification of *Shewanella oneidensis* biofilms. *Appl. Environ. Microbiol*. **72**(11):7324-7330.

Williams, B.A., Gwirtz, R.M. and Wold, B.J. (2006) Genomic DNA as a general cohybridization standard for ratiometric microarrays. *Meths Enzymol.* **410**:237-279.

Molecular, Cellular and Integrative Neuroscience

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Summary: Our laboratory investigates the psychological and neural bases of emotional and social processing, using a number of different approaches. Some studies focus at the psychological level, using behavioral data from healthy people to make inferences about how emotion modulates memory, attention, or conscious awareness. Some of these measures are quite technically innovative and have been developed in our lab: for instance, measuring peoples eye movements as they interact socially with one another.

A second approach uses neuroimaging to investigate the neural mechanisms behind emotional and social processing. The studies ask how the structure and function of the brain, in either health or disease, contributes to domains such as face processing or reward learning.

A third approach studies the performances, and the brains, of special populations. At Caltech, we have been recruiting people with agenesis of the corpus callosum to investigate the functional consequences of disruption in long-range connectivity. This work is spearheaded by Dr. Lynn Paul, and involves collaborations with Michael Tyszka and Professor John Allman. In collaboration with Joe Piven at the University of North Carolina, we have also been studying people with autism, as well as their first-degree relatives (the parents). This past year, we have been recruiting people with autism locally at Caltech as well. At the University of Iowa, we have ongoing collaborations that involve neurological populations with focal brain lesions, and that involve neurosurgical populations in whom we can record intracranially.

The two primary aims of the research are to understand how emotion contributes to aspects of human social behavior and, in so doing, to help with the diagnosis and treatment of neurological and psychiatric illnesses that involve dysfunctional social behavior. A major focus has been on two brain structures: the amygdala, and the prefrontal cortex, both structures known to participate in emotion and social cognition. We are interested in how these structures modulate memory for emotional events, how they modulate allocation of attention to emotional stimuli, and how they guide decisions about options whose outcomes have emotional value. One particularly active area of development is to better understand how amygdala and prefrontal cortex interact, and how they interface with other brain structures that also participate in emotional processing. To this end, we are developing methods for analyzing the structural and functional connectivity of the human brain.

The studies involve Caltech collaborators from diverse backgrounds. Experiments on decision-making and reward-related learning involve faculty in the Division of the Humanities and Social Sciences who apply computational models to the analysis of human decision-making, such as Professors O'Doherty, Camerer, and Rangel. Experiments on how emotion contributes to moral decisions even include faculty with backgrounds in philosophy, such as Professors Quartz and Woodward. In the biology division, we have collaborated with Professors John Allman, Christof Koch, and Shinsuke Shimojo on a variety of projects, and the investigations of the connectivity of the brain involve Michael Tyszka, Caltech's MR physicist.

187. Contributions of the amygdala to reward expectancy and choice signals in human prefrontal cortex

A.N. Hampton[1], R. Adolphs, J.M. Tyszka[2], J.P. O'Doherty[3]

The prefrontal cortex receives substantial anatomical input from the amygdala, and these two structures have long been implicated in associative learning between stimuli and outcomes, as well as between actions and their consequences. Yet little is known about how the two structures interact, especially in humans. We investigated the contribution of the amygdala to reward-related signals in prefrontal cortex in two rare subjects with focal bilateral amygdala lesions. We measured blood oxygenation-level dependent (BOLD) responses using fMRI, while the subjects performed a reversal learning task in which they first had to learn which of two choices was the more rewarding, and then flexibly switch their choice when contingencies changed. Compared to healthy controls, both subjects showed a profound change in BOLD responses in ventromedial prefrontal cortex associated with reward expectation and behavioral choice. These findings support a critical role for the human amygdala in establishing expected reward representations in prefrontal cortex, which in turn may be used to guide behavioral choice.

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Reference

Hampton, A.N., Adolphs, R., Tyszla, J.M. and O'Doherty, J.P. (2007) *Neuron*. In press.

188. Comparison of DTI fiber tracking of the monkey brain with direct tracer studies

Dirk Neumann, J. Michael Tyszka[1], Josef Parvizi[2], Ralph Adolphs

Diffusion tensor imaging (DTI) uses MRI methods to visualize white matter tracts in brains, including live human brains, and offers one of the few ways of inferring connectivity in the human brain. However, the method is very indirect, and suffers from poor spatial resolution and false positives. We have developed a probabilistic approach that estimates the direction of fibers in the brain, and have applied it at relatively high resolution to the macaque brain.

We scanned a perfusion-fixed macaque brain with contrast enhancement at 9.4 Tesla to obtain high-angular resolution diffusion images. These data were subjected to a probabilistic tractography algorithm that estimated the likelihood of pairwise connections between each of 73 functionally distinct anatomical regions of cortex. We then directly compared the connectivity matrix provided by our method to that found with direct tracer studies in visual area MT (from Lewis and van Essen) to quantify the reliability of our tractography. The comparison indicated that about 70% of connections found with tracer injections were detected using high-resolution DTI, with about a 15% error rate. When a conservative approach was applied (0% error rate), roughly a third of the known connections could be detected with complete reliability. This comparison quantifies the sensitivity and specificity of DTI approaches to investigating connectivity in the brain.

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Reference

Lewis, J.W. and Van Essen, D.C. (2000) *J. Comp. Neurol.* **428**:112-137.

189. Temporal isolation of neural processes underlying face preference decisions

Hackjin Kim, Ralph Adolphs, J.P. O'Doherty[1], Shinsuke Shimojo[2]

Decisions about whether we like someone are often made so rapidly from first impressions that it is difficult to examine the engagement of neural structures at specific points in time. We used a novel temporally extended decision-making paradigm to examine brain activation with fMRI at sequential stages of the decision-making process. Activity in reward-related brain structures, the nucleus accumbens and orbitofrontal cortex, was found to occur at temporally dissociable phases while subjects decided which of two unfamiliar faces they preferred. Choice-related signal difference in the nucleus accumbens occurred early in the trial (**Figure 1A**), during initial preference formation. Signal difference in the orbitofrontal cortex occurred late in the trial (**Figure 1B**), consistent with a role in computing the decision of which face to choose. The frontal operculum/insula was activated late also (**Figure 1C**); overall, there was a significant difference in early compared to late signals measured in the nucleus accumbens relative to the OFC. Moreover, early signal difference in the nucleus accumbens were also seen as subjects performed a control task (judging roundness of the faces), when the data were analyzed on the basis of which of those faces were later chosen as preferred. The findings support a model in which rapid, automatic engagement of the nucleus accumbens conveys a preference signal to the representation of choice in orbitofrontal cortex.

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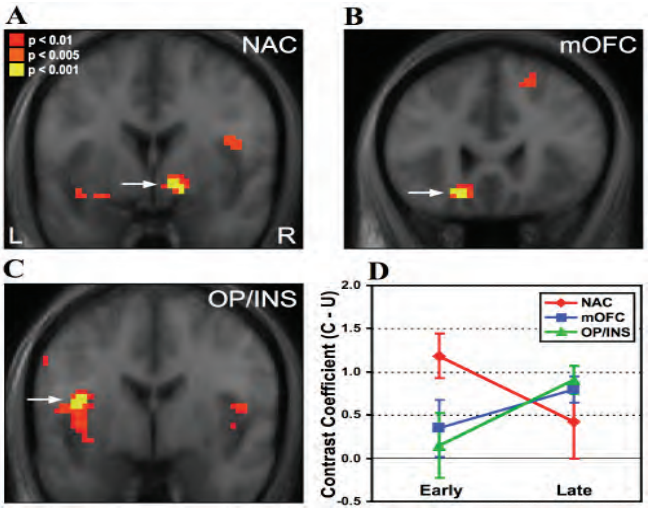

Figure 1: BOLD signal in the nucleus accumbens (NAC; A) is significantly greater for faces that were chosen as preferred compared to faces that were not preferred at early timepoint in the choice process. The same contrast in the mOFC is significant only at later timepoints (B, D). By dissociating the points in time at which these structures come into play, we were for the first time able to attribute them to specific stages of decision-making using fMRI.

190. Economic games quantify diminished sense of fairness in patients with damage to the prefrontal cortex

Ian Krabjich[1], Ralph Adolphs, Daniel Tranel[2], Natalie Denburg[3], Colin Camerer[4]

Damage to the ventromedial prefrontal cortex (VMPC) has been reliably shown to impair concern for others. Reports of the real-life behavior of patients with such damage, together with impaired performances on tasks ranging from decision-making to moral judgment to economic games, support this conclusion. Yet there is to date no numerical quantification of the deficit in terms of a general mathematical model of choice, limiting our understanding of the underlying processes that might be impaired. We quantified these social deficits using a

formal economic model of choice that incorporates a term for the disutility of unequal payoffs (fairness). We gave seven patients with focal VMPC lesions a battery of economic games that measure concern about their own monetary payoffs and about fairness. Compared to brain-damaged (N=20) and healthy (N=16) comparison subjects, the VMPC lesion patients donated significantly less money to others, and were both less trusting and less trustworthy. Pooling data across all the games, we show numerically that the VMPC patients placed significantly less weight on fair equal payoffs, and also expected less fairness on the part of others. We propose that this general insensitivity to fairness may explain impaired performance on a number of social tasks, as well as impaired social behavior in real life.

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191. Damage to the prefrontal cortex increases utilitarian moral judgments

M. Koenigs[1], L. Young[2], R. Adolphs, D. Tranel[3], F. Cushman[4], M. Hauser[5], A. Damasio[6]

The psychological and neurobiological processes underlying moral judgment have been the focus of many recent empirical studies. Of central interest is whether emotions play a causal role in moral judgment, and in parallel, how emotion-related areas of the brain contribute to moral judgment. Here we show that six patients with focal bilateral damage to the ventromedial prefrontal cortex (VMPC), a brain region necessary for the normal generation of emotions and, in particular, social emotions, produce an abnormally 'utilitarian' pattern of judgments on moral dilemmas that pit compelling considerations of aggregate welfare against highly emotionally aversive behaviors (for example, having to sacrifice one person's life to save a number of other lives). By contrast, the VMPC patients' judgments were normal in other classes of moral dilemmas. These findings indicate that, for a selective set of moral dilemmas, VMPC is critical for normal judgments of right and wrong. The findings support a necessary role for emotion in the generation of those judgments.

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Publication

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192. Using "bubbles" to determine which features of faces optimally drive BOLD response in the amygdala

M.L. Spezio, R. Adolphs

We sought to determine whether brain regions known to be responsive to faces are also selective for features within a face. We investigated this selectivity using a combination of the psychophysical method known as "bubbles" and event-related fMRI. "Bubbles" enables the sampling of facial feature space in a pseudorandom manner and shows subjects small pieces of faces on each trial. Subjects made emotional judgments from brief presentations (500 msec) of these "bubbled" faces while we measured BOLD response in the brain. Eyetracking was used to ensure that subjects were fixating and looking at the faces. We performed two types of analyses to identify the facial feature specificity of brain regions. For the first type of analysis, we quantified the amount of facial feature revealed in each image using the structural similarity index measure of visual similarity. We then conducted whole-brain analyses using the structural similarity indices for the eyes and mouth as parametric modulators in SPM2. For the second type of analysis, we used the BOLD signal in brain regions of interest as regressors to yield each region's "optimal," or classification, face. We found that the fusiform face area, the amygdala, and the anterior insula all showed greater sensitivity to the eyes compared to the mouth.

193. Fixations onto social stimuli in autism and schizophrenia

N. Sasson[1], R. Hurley[1], S. Couture[2], D. Penn[3], J. Piven[3], N. Tsuchiya, R. Adolphs

Both autism and schizophrenia feature impairments in aspects of social cognition, but it has remained unclear whether these are similar or different patterns of dysfunction. We compared the visual scanning patterns and emotion judgments of ten subjects with autism, ten with schizophrenia, and ten typically developing controls on a task in which they viewed a series of statically presented complex social scenes where faces were either included or digitally erased. All subjects were more accurate in recognizing emotions when facial expressions were included, and there were no significant differences between groups in the accuracy with which they could recognize the emotion in either type of stimulus. Total number of fixations on the images also showed no group differences. However, when analyzing fixations made onto the face region, we found a significant group X condition (face present vs. absent) interaction: controls increased their viewing time on the face region when faces were present to a significantly greater degree than both the autism and schizophrenia groups. Furthermore, while both the typical and the schizophrenia groups oriented to face regions faster when faces were present, the autism group showed no such distinction. In total, the findings suggest that individuals with autism or schizophrenia are not drawn to fixate faces in complex social scenes to the same degree as controls, and hence

may not utilize facial information in a typical manner when assessing the emotional content of a social scene.

Figure 2: Scanpaths from a healthy control subject (top row), a subject with autism (middle row), and a subject with schizophrenia (bottom row) showing representative fixation patterns onto the stimuli.
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Reference

Sasson, N., Tsuchiya, N., Hurley, R., Couture, S.M., Penn, D.L., Piven, J. and Adolphs, R. (2007) *Neuropsychologia* **45**:2580-2588.

194. Quantification of morphometric and connectivity abnormalities in agenesis of the corpus callosum

L.K. Paul, J.M. Tyszka[1], M. Marti[2], D. Kahn, R. Adolphs

We have continued our studies of people with agenesis of the *corpus callosum* and expanded investigations of the structural abnormalities of their brains. One project is quantifying the volumes of various regions of the brain; another is measuring white-matter connectivity. Using diffusion imaging approaches (see abstract 188), we are visualizing and quantifying abnormal connectivity in these brains (**Figure 3**; also see cover).

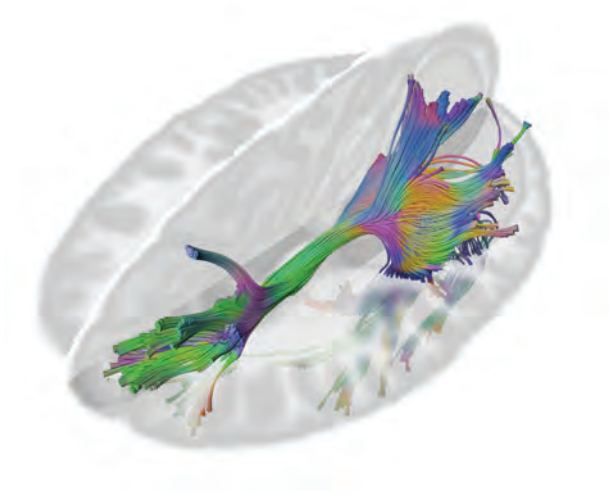

Figure 3: Probst bundles in agenesis of the corpus callosum (image courtesy of J.M. Tyszka). A patient with agenesis of the corpus callosum was scanned in Caltech's 3T scanner. Diffusion imaging data were then subjected to a tractography algorithm that visualizes likely macroscopic fiber paths in the brain. The image shows saggital and horizontal planes of the brain (the front of the brain is towards the bottom left of the image), and colored fibers are likely connections. These connections, called "Probst bundles" are highly abnormal and likely arise from misrouted callosal axons. They run anterior-posterior along the medial wall of each hemisphere (the Prost bundle in the left hemisphere is shown here). Different colors encode different orientations of the bundle.
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Publications

Adolphs, R. (2006) How do we know the minds of others? Domain specificity, simulation, and enactive social cognition. *Brain Res*. **1079**:25-35.

Adolphs, R. (2006) Perception and emotion: How we recognize facial expressions. *Curr. Direct. Psychol. Sci.* **15**:222-226.

Buchanan, T.W., Tranel, D. and Adolphs, R. (2006) Impaired memory retrieval correlates with individual differences in cortisol response but not autonomic response. *Learning & Memory* **13**:382-387.

Buchanan, T.W., Tranel, D. and Adolphs, R. (2006) Memories for emotional autobiographical events following unilateral damage to medial temporal lobe. *Brain* **129**:115-127.

Gosselin, N., Peretz, I., Johnsen, E. and Adolphs, R. (2006) Amygdala damage impairs emotion recognition from music. *Neuropsychologia* 2006 Sep 11 epub.

Gosselin, N., Samson, S., Adolphs, R., Noulhiane, M., Roy, M., Hasboun, D., Baulac, M. and Peretz, I. (2006) Emotional responses to unpleasant music correlates with damage to the parahippocampal cortex. *Brain* 2006 Sep 7 epub.

Hampton, A.N., Adolphs, R., Tyszka, J.M. and O'Doherty, J.P. (2007) Contributions of the amygdala to reward expectancy and choice signals in human prefrontal cortex. *Neuron*. In press.

Koenigs, M., Young, L., Adolphs, R., Tranel, D., Cushman, F., Hauser, M. and Damasio, A. (2007) Damage to the prefrontal cortex increases utilitarian moral judgments. *Nature* **446**:908-911.

Neumann, D., Spezio, M.L., Piven, J. and Adolphs, R. (2006) Looking you in the mouth: Abnormal gaze in autism resulting from impaired top-down modulation of visual attention. *Social Cogn. Affect. Neurosci.* **1**:194-202.

Paul, L.K., Brown, W.S., Adolphs, R., Tyszka, J.M., Richards, L.J., P. Mukherjee, P. and Sherr, E.S. (2007) Agenesis of the corpus callosum: Genetic, developmental, and functional aspects of connectivity. *Nature Reviews Neurosci*. **8**:287-299.

Sasson, N., Tsuchiya, N., Hurley, R., Couture, S.M., Penn, D.L., Piven, J. and Adolphs, R. (2007) Orienting to social stimuli differentiates social cognitive impairment in autism and schizophrenia. *Neuropsychologia* **45**:2580-2588.

Spezio, M.L. Huang, P-Y.S., Castelli, F. and Adolphs, R. (2007) Amygdala damage impairs eye contact during conversations with real people. *J. Neurosci*. **27**:3994-3997.

Spezio, M.L., Adolphs, R., Hurley, R.S.E. and Piven, J. (2007) Abnormal use of facial information in high functioning autism. *J. Autism & Dev. Disorders* **37**:929-939.

Spezio, M.L., Adolphs, R., Hurley, R.S.E. and Piven, J. (2007) Analysis of face gaze in autism using "bubbles." *Neuropsychologia* **45**:144-151.

Tranel, D., Gullickson, G., Koch, M. and Adolphs, R. (2006) Altered experience of emotion following bilateral amygdala damage. *Cogn. Neuropsych*. **11**:219-232.

Tsuchiya, N., and Adolphs, R. (2007) Emotion and consciousness. *Trends Cogn. Sciences* **11**:158-167.

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195. Von Economo Neuron (VEN) stereological counts in FI and ACC

John Allman, Jason Kaufman, Nicole Tetreault, Atiya Hakeem, Kebreten Manaye[1], Katerina Semendeferi[2]

We have compared the number of VENs in layers 3 and 5 with the total number of neurons in these layers in FI in apes and in humans of different ages. These data show that the percentage of VENs is consistently about 2% of the total neurons in adult apes whereas the average is only about 1.2% in adult humans. The VEN percentages in FI undergo an interesting ontogeny in humans. Before 34 weeks post conception, there are no morphologically identifiable VENs in FI. At birth, the VENs comprise less than 1% of the total neurons, but that fraction increases rapidly so that by eight months it exceeds 5% and remains at this level for the next three years or more. Then the percentage declines until it reaches a little over 1% in adults. This raises the question as to what happens to these VENs? Do they undergo apoptosis and die or are they transformed into another cell type? During the course of development the absolute number of VENs increases dramatically after birth to reach a maximum at 8 months and then appears to decline during childhood. The absolute number of VENs is very high for the 8 month-old infant, which may be an outlier, but expressed as a percentage of total neurons, the 8 month-old is in line with 19 month and 4 year-old VEN populations expressed as a percentage of the total number of neurons in layers 3 and 5 of FI. When the number of VENs in FI is compared between the right and left hemispheres, we found that the right hemispheric specialization emerges postnatally in humans and that there are consistently about 30 to 40% more VENs in the right hemisphere in apes. The ape data imply that the right hemispheric specialization for the social emotion/cognition emerged in the common ancestors of apes and humans long before the advent of language.

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196. Von Economo-like neurons in an African elephant

Atiya Hakeem, Chet Sherwood[1], Chris Bonar[2], Patrick Hof[3], John Allman

We have obtained two brains of African elephants from the Cleveland Zoo and have begun a histological analysis of the cerebral cortex. We have found VEN-like neurons in anterior cingulate cortex (ACC), several small loci near the frontal pole, and in a region that is similar in topographic location to fronto-insular cortex (FI) in the human brain. We have made a stereological count of the VENs in FI in the right hemisphere and found an estimated population of 10,150. These cells comprise 0.78% of the total neuron population of the VEN containing layers. The number of VENs in FI in the elephant is within the range we have observed in apes, although the percentage of total neurons is somewhat lower. The figure illustrates the location of area FI in section number 570, and the inset shows the location of each VEN (red dots) in FI in this section. Area FI in humans is activated by the discrimination of intention in faces (Baron-Cohen *et al.*, 1999). This specialization could be related to the capacity to recognize one's own face in a mirror image (mirror self-recognition) that can be done by great apes and humans. Recently, the capacity for mirror self-recognition was found in elephants (Plotnik *et al*., 2006) and it is tempting to speculate that FI is involved in this capacity in elephants.

One of the most important questions arising from this finding is whether the VENs in elephants are a specialization that arose independently from the VENs in hominoids and in whales where they have also been found. Elephants are members of the Afrotheria, a group of genetically related but morphologically diverse mammals which originated in Africa, and which also includes the hyraxes, sirenia, aardvarks, elephant shrews, golden moles, and tenrecs. The next most closely related group of mammals are the Xenarthia (armadillos, anteaters, and sloths). We have examined serial sections through the brains of a hyrax, a manatee (sirenia), an anteater, and a sloth and have not found any VENs in FI and ACC in these mammals. Our observations suggest that the VENs are an independent specialization in elephants as they also appear to be in whales.

A possible interpretation of these data is that there exists a population of neurons in layers 3 and 5 of anterior cingulate and fronto-insular cortex that may be present in all mammals and is genetically distinct from other neuronal populations in these areas; in mammals with very large brains the developmental antecedent parts of this population were transformed by a series of mutations into VENs. These transformations may serve as fast relays of information from these structures to other parts of these very large brains as part of a suite of adaptations supporting complex social behavior.

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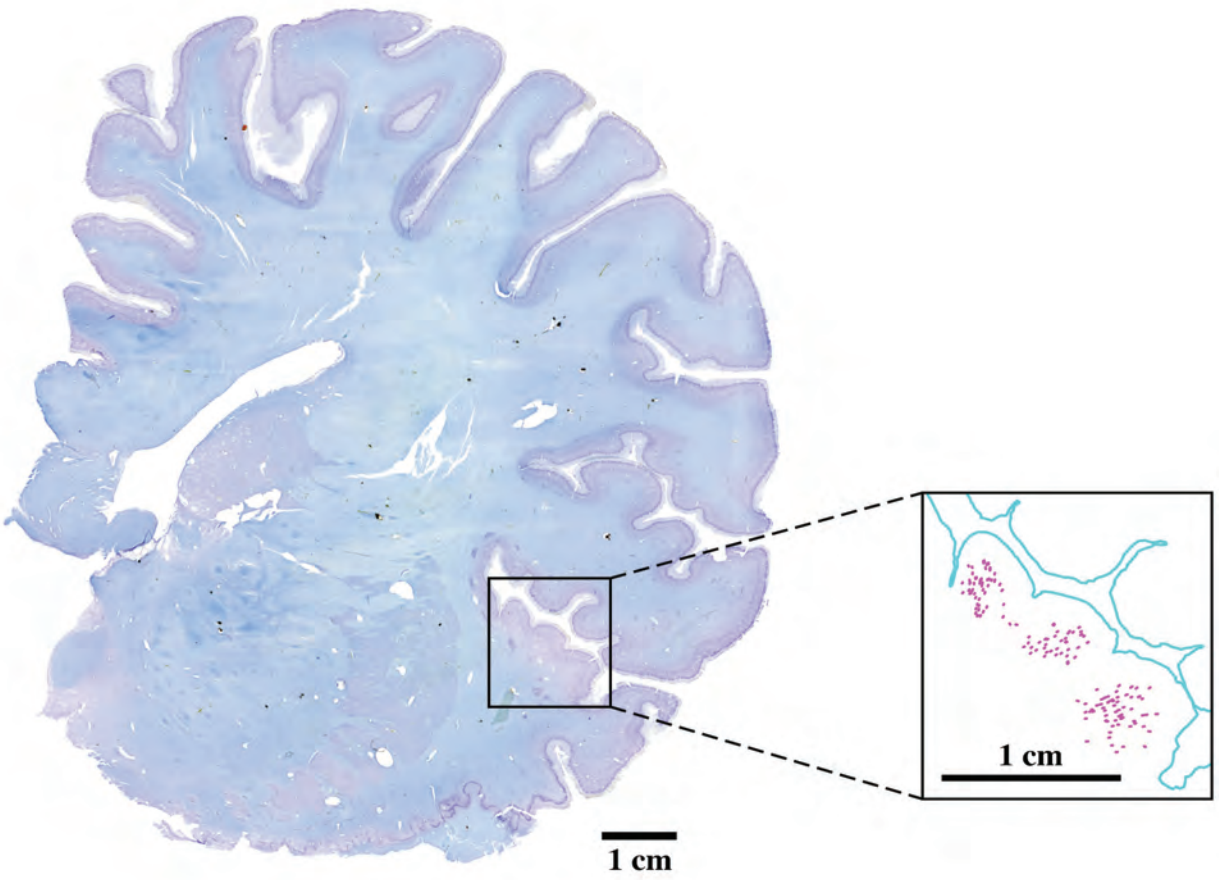
References

Baron-Cohen, S., Ring, H., Wheelwright, S., Bullmore, E., Simmons, A. and Williams, S. (1999) *European J. Neurosci*. **11**:1891-1898.

Plotnik, J., deWaal, F. and Reiss, D. (2006) *Proc. Natl. Acad. Sci. USA* **103**:17053-17057.



Publications

Seeley, W.W., Carlin, D.A., Allman, J.M., Macedo, M.N., Bush, C., Miller, B.L. and Dearmond, S.J. (2006) Early frontotemporal dementia targets neurons unique to apes and humans. *Ann. Neurol.* **60**(6):660-667. http://www.allmanlab.caltech.edu/PDFs/Seeley2006.pdf

Woodward, J. and Allman, J.M. (2007) Moral intuition: Its neural substrates and normative significance. *J. Physiol., Paris.* In press. http://www.allmanlab.caltech.edu/PDFs/WoodwardAllman2007.pdf

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Summary:
Neural mechanisms for visual-motor integration, spatial perception and motion perception
While the concept of artificial intelligence has received a great deal of attention in the popular press, the actual determination of the neural basis of intelligence and behavior has proven to be a very difficult problem for neuroscientists. Our behaviors are dictated by our intentions, but we have only recently begun to understand how the brain forms intentions to act. The posterior parietal cortex is situated between the sensory and the movement regions of the cerebral cortex and serves as a bridge from sensation to action. We have found that an anatomical map of intentions exists within this area, with one part devoted to planning eye movements and another part to planning arm movements. The action plans in the arm movement area exist in a cognitive form, specifying the goal of the intended movement rather than particular signals to various muscle groups.

Neuroprosthetics

One project in the lab is to develop a cognitive-based neural prosthesis for paralyzed patients. This prosthetic system is designed to record the electrical activity of nerve cells in the posterior parietal cortex of paralyzed patients, interpret the patients' intentions from these neural signals using computer algorithms, and convert the "decoded" intentions into electrical control signals to operate external devices such as a robot arm, autonomous vehicle or a computer.

Recent attempts to develop neural prosthetics by other labs have focused on decoding intended hand trajectories from motor cortical neurons. We have concentrated on higher-level signals related to the goals of movements. Using healthy monkeys with implanted arrays of electrodes we recorded neural activity related to the intended goals of the animals and used this signal to position cursors on a computer screen without the animals emitting any behaviors. Their performance in this task improved over a period of weeks. Expected value signals related to fluid preference, or the expected magnitude or probability of reward were also decoded simultaneously with the intended goal. For neural prosthetic applications, the goal signals can be used to operate computers, robots and vehicles, while the expected value signals can be used to continuously monitor a paralyzed patient's preferences and motivation.

Movable probes

In collaboration with Joel Burdick's laboratory at Caltech, we have developed a system that can autonomously position recording electrodes to isolate and maintain optimal quality extracellular recordings. It consists of a novel motorized microdrive and control algorithm. The microdrive uses very small piezoelectric actuators that can position electrodes with micron accuracy over a 5 mm operating range. The autonomous positioning algorithm is designed to detect, align, and cluster action potentials, and then command the microdrive to move the electrodes to optimize the recorded signals. This system has been shown to autonomously isolate single unit activity in monkey cortex. In collaboration with Yu-Chong Tai's lab and the Burdick lab we are now developing an even more compact system which uses electrolysis-based actuators designed to independently move a large number of electrodes in a chronically implanted array of electrodes.

Coordinate frames

Our laboratory also examines the coordinate frames of spatial maps in cortical areas of the parietal cortex coding movement intentions. Recently, we have discovered that plans to reach are coded in the coordinates of the eye. This is a particularly interesting finding because it means the reach plan at this stage is still rather primitive, coding the plan in a visual coordinate frame rather than the fine details of forces for making the movement. We have also discovered that when the animal plans a limb movement to a sound, this movement is still coded in the coordinates of the eye. This finding indicates that vision predominates in terms of spatial programming of movements in primates.

We have also been examining the coordinate frame for coordinated movements of the hand and eyes. In the dorsal premotor cortex we find a novel, "relative" coordinate frame is used for hand-eye coordination. Neurons in this cortical area encode the position of the eye

to the target, the position of the hand to the target, and the relative position of the hand to the eye. A similar relative coding may be used for other tasks which involve the movements of multiple body parts such as bimanual movements.

Motion perception

Another major effort of our lab is to examine the neural basis of motion perception. One series of experiments is determining how optic flow signals and efference copy signals regarding eye movements are combined in order to perceive the direction of heading during self-motion. These experiments are helping us understand how we navigate as we move through the world. A second line of investigation asks how motion information is used to construct the three dimensional shape of objects. We asked monkeys to tell us which way they perceived an ambiguous object rotating. We found an area of the brain where the neural activity changed according to what the monkey perceived, even though he was always seeing the same stimulus. In other experiments we have been examining how we rotate mental images of objects in our minds, so-called mental rotation. In the posterior parietal cortex we find that these rotations are made in a retinal coordinate frame, and not an object based coordinate frame, and the mental image of the object rotates through this retinotopic map.

Local field potentials

The cortical local field potential (LFP) is a summation signal of excitatory and inhibitory dendritic potentials that has recently become of increasing interest. We have reported that LFP signals in the saccade and reach regions provide information about the direction of planned movements as well as the state of the animal; e.g., baseline, planning a saccade, planning a reach, executing a saccade, or executing a reach. This new evidence provides further support for a role of the parietal cortex in movement planning. It also shows that LFPs can be used for neural prosthetics application. Since LFP recordings from implanted arrays of electrodes are more robust and do not degrade as much with time compared to single cell recordings, this application is of enormous practical importance.

fMRI in monkeys

We have successfully performed functional magnetic resonance imaging (fMRI) experiments in awake, behaving monkeys. This development is important since this type of experiment is done routinely in humans and monitors the changes in blood flow during different cognitive and motor tasks. However, a direct correlation of brain activity with blood flow cannot be achieved in humans, but can in monkeys. Thus, the correlation of cellular recording and functional MRI activation in monkeys will provide us with a better understanding of the many experiments currently being performed in humans. A 4.7 Tesla vertical magnet for monkey imaging has recently been installed in the new imaging center in the Broad building. We are using this magnet, combined with neural recordings, to examine the correlation between neural activity and fMRI signals.

197. The posterior parietal cortex encodes the first and second goal in sequential reaching arm movements

Daniel Baldauf, He Cui, Richard A. Andersen

The parietal reach region (PRR) has been found to be involved in the preparation of goal-directed arm movements to single targets. In this study we explored whether the reach planning activity in this region encodes only the target of the very next movement or, alternatively, also a subsequent goal in a fast reaching sequence. We trained two macaque monkeys to perform sequential reaches to peripheral positions without breaking eye fixation. While the monkey initially positioned his hand at a circle in the center of the touch screen a small triangle and square were presented in the periphery for 400 ms and cued the first and second movement goal, respectively. After these cues disappeared the animal had to memorize the locations of the two targets for 600 ms and to prepare for a rapid double reach sequence. Once the central circle turned off (GO-signal) the animals had to reach in a predetermined order first to the location where the little square had been presented and then immediately to the location where the triangle had been. The movement goals were arranged such that either the target for the first reach or the target for the second reach was inside the response field of an isolated neuron. We analyzed the neural activity of single cells in PRR during the last 400 ms of the memory period between cue offset and the GO-signal. Most cells in PRR encoded the first as well as the second reach of the planned reaching sequence in advance of movement initialization, although the response for the second target is usually less than for the first. Among the 43 neurons recorded so far 31% were only active when the first target was inside the cell's response field and 10% only if the second, final goal location lay in the cell's preferred direction. The majority of cells (59%), however, showed significantly elevated activity during the late delay period for either target in the response field.

The results indicate that the posterior parietal cortex is involved in the spatial planning of more complex action patterns and represents immediate and subsequent movement goals.

198. Neural representation of sequential states within an instructed task

Michael Campos, Boris Breznen, Richard A. Andersen

In the study of the neural basis of sensorimotor transformations, it has become clear that the brain does not always wait to sense external events and afterwards select the appropriate responses. If there are predictable regularities in the environment, the brain begins to anticipate the timing of instructional cues and the signals to execute a response, and even the consequences of actions. An organism's ability to anticipate events reveals an internal representation of the sequential progression of behavioral states within the context of the task being performed. Using the same eye movement tasks while

recording neural data from two cortical oculomotor areas in the rhesus monkey, we found complementary spatial and sequential state representations of the Lateral Intraparietal Area (LIP) and the Supplementary Eye Field (SEF). While both areas encoded the position of eye movement targets, this spatial encoding was more consistently found in single neurons of LIP. In addition, the neurons of the SEF were found to collectively encode the progression of behavioral states of the task, with individual neurons detecting and/or anticipating different events or sets of events in the task or becoming tonically activated or depressed from one event to another and thus encoding states in an event-based manner. The entirety of responses from SEF was used to decode the current temporal position within the context of the task. Since LIP neurons were found to respond similarly when encoding an eye movement plan (saccade period) or the location of brightly flashed stimulus (cue period), the temporal information provided from SEF could be used to imply the significance of the spatial representation found in LIP.

199. Posterior parietal cortex encodes autonomously selected motor plans during effector choice

He Cui, Richard A. Andersen

The posterior parietal cortex (PPC) of rhesus monkeys has been found to encode cognitive set regarding task rules and behaviorally relevant categories of sensory stimuli. Two of its subdivisions, the lateral intraparietal area (LIP) and the parietal reach region (PRR) exhibit persistent activity when monkeys are instructed to perform delayed saccades and delayed reaches, respectively. To address whether such task-selective activity prospectively encodes impending movements or retrospectively represents past sensory cues instructing effectors, we examined PPC activity while monkeys chose to acquire a target either by saccading or reaching in the absence of direct sensory instructions specifying the effectors. The monkey was seated in front of a board with an array of buttons, each containing a red and a green LED. At the beginning of each trial, both the green and red LEDs in the central button were turned on and the monkey was required to fixate and touch it. Then both the red and green LEDs in a peripheral button were turned on simultaneously, and the monkey was required to continue fixating and touching the central fixation spot until it disappeared (GO signal). After 600 ms of cue duration, the green (red) LED was turn off and only the red (green) remained on in 25% of trials, instructing a saccade (reach) after a delay of 600 ms. In the remaining 50% of the trials, both peripheral LEDs extinguished, and the monkey could decide to either shift gaze after the delay to the location of the peripheral target while continuing to touch the center spot, or keep fixation of the center spot but move the arm to reach to the target. In the effector choice trials, the monkey was rewarded only if his choice matched the computer's choice and computer biased its choice against the monkey's choice sequence. This competitive algorithm provided an effective method to balance the monkey's bias for saccade/reach selection. We recorded 100 LIP cells and 91 PRR cells from two monkeys. The results demonstrated that the PPC still shows effector specificity for autonomously chosen saccades and reaches, with LIP preferential for saccades and PRR preferential for reaches. Differential activity associated with non-spatial decisions regarding effector choice suggests that the cognitive functions of the PPC include not only passively transforming sensory stimuli to behaviorally relevant representations, but also an active role in plan selection and movement preparation.

200. Examination of local field potential in the parietal reach region during a free-choice self-paced reach

EunJung Hwang, Richard A. Andersen

Recent studies have shown that the local field potential (LFP) oscillation in the 20-40 Hz band in the parietal reach region (PRR) is tuned to the upcoming reach direction. It has also been found that the LFP spectral structure changes when the behavioral state shifts from movement planning to execution; i.e., oscillation power at 20-40 Hz decreases and power below 10 Hz increases. However, this spatial tuning and event state coding were studied under only one experimental condition an instructed delayed reach task, leaving open the question of the tuning and structure of the LFPs in other experimental conditions. Here, we examined LFP characteristics in an experiment that is one step closer to the real life situation, a free choice self paced reach task. In this task, eight equally spaced visual stimuli were presented around a circle and stayed on until the end of the trial. The monkey chose a reach among these eight targets and he also chose when to initiate the movement without any external cue. First, in the free choice task, the LFP oscillation power at 20-40 Hz was still enhanced during the planning period compared to the baseline or movement periods, consistent with the idea that the oscillation is not associated with memorization of the target location. However, the spatial tuning to the upcoming reach direction in this enhanced LFP oscillation was much less than in the single target situation. The tuning curves were much shallower, suggesting a competitive interaction between the multiple targets. Interestingly, the spatial tuning of the spikes remained largely unchanged. This dissociation is consistent with the spikes representing largely the output of a visuomotor transform performed in PRR and the LFPs representing the synaptic inputs and intercortical processing. Secondly, the spectral structure robustly changed from planning to execution in the self paced reach task, confirming that this change in the LFP reflects a switch of the internal event state instead of visually-evoked potentials.

201. Decoding a 'go' signal using the local field potential in the parietal reach region

EunJung Hwang, Richard A. Andersen

In the parietal reach region, the spectral structure of the local field potential (LFP) is uniquely associated with internal event states. During reach planning, the 20-40 Hz band is enhanced while the lower frequency band (<10 Hz) is depressed. As the behavioral state changes from planning to execution, this structure reverses. In this

study, we show that in macaque, the spectral structure reversal can be trained to control the initiation of the cursor movement during brain control trials. The time derivative of the difference in LFP power between the 20-40 Hz and 1-10 Hz bands was continuously monitored. When this time derivative exceeded a preset threshold ('go' signal), the cursor was jumped from the center to the periphery of the screen. In order to be rewarded, the monkey needed to learn to generate the 'go' signal after a predetermined hold period, which was assigned in a blockwise manner. Initially, the monkey generated the 'go' signal too early or could not generate it at all without initiating the actual reaching movement. However, he quickly learned to manipulate the LFP to generate a robust 'go' signal after the predetermined hold period and without physical movement, reaching a near perfect performance level.

202. Discrete cognitive state decoding, with applications to neural prosthetics

Nick Hudson, Joel Burdick, Richard A. Andersen

A novel method to identify mathematical models of neural activity that relate to discrete temporal changes of behavioral or cognitive state is presented. This work is motivated by its potential application to cognitive neural prosthetics, where a "supervisory decoder" is required to classify the activity of multi-unit extracellular recordings into suitable discrete modes, such as: (1) the patient is disinterested in using the prosthetic; (2) the patient wishes to use the prosthetic; (3) the patient is planning an action that must be decoded; (4) the patient wants to execute the planned action; (5) the patient wants to scrub or change the current action. We introduce a Gibbs sampling method to identify the key parameters of a GLHMM, a hybrid dynamical system that combines a Generalized Linear Model (GLM) for the neuronal signals with a Hidden Markov Model (HMM) that describes the discrete transitions between cognitive or planning states. Both local field potentials (LFP) and spike arrival times can be modeled in a unified way with the Generalized Linear Models (GLM). Once the parameters of the model are identified, it can be used as the basis for the supervisory decoding of the current cognitive or planning state. This modeling and decoding framework is compared to a standard HMM trained with the Baum-Welch algorithm, and is then demonstrated on a simulated example to illustrate its characteristics. The identification algorithm is then applied to extracellular neural activity recorded from multi-electrode arrays chronically implanted in the Posterior Parietal cortex of a rhesus monkey. The results demonstrate the ability to accurately decode changes in behavioral or cognitive during tasks which simulate a neural prosthetic, even when trained on small data sets.

203. Time-courses of fMRI BOLD signals in frontal and parietal cortex reflect monkeys' decisions in a free-choice oculomotor task

Igor Kagan, Axel Lindner, Asha Iyer, Shawn Wagner, Richard A. Andersen

Studies of decision making in primates typically focus on externally guided choices, employing perceptual judgment and reward reinforcement. However, internally-generated decisions and biases are also important for behavior. We investigated the neural substrates of motor planning and voluntary decision-making in monkeys with fMRI using a high-field 4.7T vertical primate scanner. We have previously shown strong contralateral tuning of cue and mnemonic/preparatory delay BOLD activity preceding memory saccades in frontal and parietal cortex. Building on this research, we designed a new free-choice task. Two randomly selected spatial cues, to the left and to the right of fixation, were presented simultaneously for 200 ms and after a long delay the monkeys had to make a memory saccade to either of the two cued locations. The reward was not contingent on the monkeys' choices but was delivered for each properly finished trial. Both monkeys in this experiment exhibited a rightward bias, but still chose left targets in 35% of trials. The mean saccadic latency difference was only minimally (8 ms) longer in free choice than in instructed (one cue only) trials, suggesting that monkeys decided about the direction of the upcoming movement before the end of the delay period.

We used an event-related analysis to separate cue, delay and saccade activity and compared BOLD time-courses in free-choice and instructed-memory saccade tasks in functionally defined cortical regions. In frontal cortex, the transient cue response was not spatially tuned in the free choice task, implying that activity in the early cue period does not contribute to the decision processing and response selection. Following the initial transient, activity in dlPFC and FEF separated early and increased through the memory period in trials where monkeys selected a contralateral target, relative to the ipsilateral trials. In contrast, the contralateral delay activity in PMd diverged from the ipsilateral in the late phase of the delay period. In parietal cortex, area LIP exhibited contralateral tuning already in the cue response and there was a gradual increase of selectivity during the delay period. In the superior temporal sulcus, a few regions showed stronger contralateral delay activity in the instructed, but not the free-choice task. In all areas, the contralateral delay activity in free choice trials was smaller than in instructed memory trials, while the ipsilateral activity was often higher in free-choice trials. The contralateral activity build-up in the free-choice task demonstrates that frontal and parietal areas participate, but contribute differently, in the decision process.

204. Expected reward magnitude modulates fMRI activity in monkey ventral and dorsal cortical streams and the striatum during a goal-directed saccade task

Axel Lindner, Igor Kagan, Asha Iyer, J.P. O'Doherty, W. Schultz, Richard A. Andersen

One of the key questions in decision making is how goal-directed actions are selected based on the expected utility with which they are associated. In order to capture the networks subserving this mechanism we employed whole brain event-related fMRI in three alert monkeys performing a visually-guided saccade task. In this task monkeys fixated a central spot until the saccade target appeared in the periphery. Monkeys had to saccade towards the target and afterwards had to maintain central fixation for 10 sec in order to obtain reward. The magnitude of reward was indicated to the monkeys by the color of the target: it either predicted a large amount (33% of trials) or a small amount of liquid reward (33% of trials). In the remaining third of the randomly interleaved trials both targets were presented simultaneously and monkeys were free to choose either of the two. In these trials all monkeys consistently saccaded towards the target predicting the large reward. This preference was also reflected by shorter saccadic latencies and higher performance rates in "large reward" as compared to "small reward" trials. In line with these behavioral indicators of preference, fMRI-activity along the ventral cortical stream (V4, STS), the dorsal cortical stream (LOP/LIP and FEF), and the striatum was significantly higher during target presentation and/or saccade execution in the "large reward" trials. Furthermore, the striatum and areas within the STS exhibited sustained activity in expectancy of the delayed reward during the 10 sec waiting period. The level of this activity was higher for larger reward magnitudes. It also clearly differed in time-course from motor and somatosensory activity due to licking in anticipation of reward. Finally, we reversed the object-reward association while scanning one monkey during learning of this new association. During the initial stages of learning the monkey continued to choose the previously preferred, now "small reward" target. This "wrong" preference was reflected in STS-activity while differential fMRI-activity within the striatum vanished. During subsequent stages of learning the size of reward was again "correctly" reflected by activity within the striatum. This new association then became apparent in the monkey's behavior, namely "correct" choices and shorter saccadic latencies in "large reward" trials. The activity within the STS changed in step with the behavioral changes. In summary our results emphasize a major role of the striatum in reward learning. Furthermore, they demonstrate that the cortical representations of visual action goals (V4, STS, LOP/LIP) and action plans (LIP, FEF) are modulated by expected reward magnitude.

205. Modeling perceptual ambiguity resolution in V1

Zoltan Nadasdy, Richard A. Andersen

Recording single unit activity from V1 of the primate brain during a perceptual decision task uncovered two classes of neurons with opposite perceptual biases. One class of neurons increased the firing rate when the percept was consistent with the neuron's tuning (correlated neurons). The other class of neurons decreased the firing rate under the same condition (anti-correlated neurons). Although anti-correlated neuronal responses have been observed in other cortical areas, the functional segregation of correlated and anti-correlated neurons is lacking explanation. We performed Monte-Carlo simulations to reproduce the perceptual bias that was observed *in vivo*. Our model implemented a three-layer recurrent network consisting of a sensory input layer (V1), an integrative layer (MT) and a putative higher cortical area engaging in the perceptual decision task. Feedback connections from MT and from the higher cortical area were projected back to V1. The perceptual task involved an alternative forced-choice decision about a structure-from-motion stimulus cued by motion direction and disparity. Accordingly, V1 neurons were modeled by a combination of direction and disparity tuning. Single unit activity was modeled by a modulated Poisson process. The baseline firing rate of neurons was convolved by time-dependent kernels corresponding to individual bottom-up and top-down biases. We compared the choice probability from the network with that of the single unit recording data using ROC analysis under ambiguous stimulus conditions, when perceptions of the two alternative interpretations were bistable, so that the contribution of neuronal biases to the ambiguity resolution was unaffected by the visual input. The model demonstrated that the occurrence of anti-correlated neurons is expected based on the random combination of disparity and direction tuning. Furthermore, the model replicated time-dependent population biases of neuronal activity, consistent with experimental data. We concluded that a significant perceptual bias may derive from V1 and that this bias does not require feedback from higher visual areas. However, the time-dependent fluctuation of the perceptual bias, observed in single unit recordings, does require feedback connections from higher visual or decision-making areas to be able to influence subsequent perceptual decisions.

206. The Caltech Neural Prosthetic System: Design and performance

Cevat Ustun, Jeremy Emken, Dan Rizzuto, Richard A. Andersen

The Caltech Neural Prosthetic (CNP) system is designed to provide novel communication capabilities to people living with severe paralysis and we anticipate recruiting participants for clinical trials next year. The CNP system is comprised of three different subsystems: (1) the CNP implant, which utilizes arrays of Parylene-coated, platinum/iridium microelectrodes implanted into parietal cortex to record high-resolution neural activity, (2) the CNP computer apparatus, which connects to the implant and decodes the user's neural activity, providing

them with control over a various software applications; and (3) the CNP insertion tool, which is used to precisely target the implantation of the electrode arrays into human parietal cortex. We have quantified the performance of the CNP system using a battery of mechanical and electrical tests. Performance metrics include cortical dimpling, electrode stability, component mechanical integrity, and channel crosstalk. As part of the system design process we have also surveyed participants with severe paralysis to assess their preferences regarding software application functionality. In addition to presenting results from the survey, we will also provide a roadmap for transitioning the CNP system to clinical trials.

Publications

Bokil, H., Pesaran, B., Andersen R.A. and Mitra, P. (2006) A method for detection and classification of events in neural activity. IEEE Transactions of Biomedical Engineering, **53**(8):1678-1687.

Branchaud, E.A., Andersen, R.A. and Burdick, J.W. (2006) An algorithm for autonomous isolation of neurons in extracellular recordings. Proceedings of the 2006 IEEE International Conference on Biomedical Robotics and Biomechatronics.

Brozovic, M. and Andersen R.A. (2006) A nonparametric quantification of neural response field structures. *NeuroReport* **17**(10):963-967.

Brozovic, M., Gail, A. and Andersen, R.A. (2007) Gain mechanism for contextually guided visuomotor transformations. *J. Neurosci*. **27**(39):10588-10596.

Buneo C.A. and Andersen, R.A. (2006) The posterior parietal cortex: Sensorimotor interface for the planning and online control of visually guided movements. *Neuropsychologia* **44**:2594-2606.

Cui, H. and Andersen, R.A. (2007) Posterior parietal cortex encodes autonomously selected motor plans. *Neuron*. In press.

Gail, A. and Andersen, R.A. (2006) Neural dynamics in monkey parietal reach region reflect context-specific sensorimotor transformations. *J. Neurosci.* **26**(27):9376-9384.

Greger, B., Kateb, B., Gruen, P. and Patterson, P.H. (2007) A chronically implantable, hybrid cannula-electrode device for assessing the effects of molecules on electrophysiological signals in freely behaving animals. *J. Neurosci. Meths*. **163**:321-325.

Huang, R., Pang, C., Tai, Y.C., Emken, J., Ustun, C., Rizzuto, D.S. and Andersen, R.A. (2007) Parylene coated silicone probes for neural prosthetics. IEEE International Conference of Nano/Micro Engineered and Molecular Systems. In press.

Lee, B., Pesaran, B. and Andersen, R.A. (2007) Translation speed compensation in the dorsal aspect of the medial superior temporal area. *J. Neurosci.* **27**(10):2582-2591.

Musallam S., Bak M.J., Troyk P.R. and Andersen, R.A. (2007) A floating metal microelectrode array for chronic implantation. *J. Neurosci. Meths*. **160**:122-127.

Nenadic, Z., Rizzuto, D.S., Andersen, R.A. and Burdic, J.W. (2007) Advances in cognitive neural prosthesis: Recognition of neural data with an information-theoretic onjective toward brain-computer interfacing. Dornhege, G., Millan, J.R., Hinterberger, T., McFarland D.J., Muller, K.-R. (Eds.) The MIT Press, Cambridge, Massachusetts, London, England, pp.175-190.

Pang, C., Tai, Y-C., Burdick, J.W. and Andersen R.A. (2006) Electrolysis-based diaphragm actuators. *Nanotechnology* **17**:S64-S68.

Pesaran, B., Musallam, S. and Andersen, R.A. (2006) Cognitive neural prosthetics. *Curr. Biol.* **16**:(2):77-80.

Pesaran, B., Nelson, M. and Andersen R.A. (2006) Dorsal premotor neurons encode the relative position of the hand, eye, and goal during reach planning. *Neuron* **51**:125-134.

Quiroga, R.Q., Snyder, L.H., Batista, A.P., Cui, H. and Andersen, R.A. (2006) Movement intention is better predicted than attention in the posterior parietal cortex. *J. Neurosci.* **26**(13):3615-3620.

Sarpeshkar, R., Wattanapanitch, W., Rapoport, B.I., Arfin, S.K., Baker, M.W., Mandal, S., Fee, M.S., Mussalam, S. and Andersen, R.A. (2007) Lower-power circuit for brain-machine interfaces. IEEE International Symposium on Circuits and Systems, New Orleans.

Scherberger H. and Andersen, R.A. (2007) Target selection signals for arm reaching in the posterior parietal cortex. *J. Neurosci*. **27**(8):2001-2012.

Torres, E. and Andersen, R.A. (2006) Space-time separation during obstacle-avoidance learning in monkeys. *J. Neurophysiol*. **96**:2613-2632.

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Summary: There are currently three major areas of investigation in this laboratory: the development of neural stem cells; the functional neuroanatomy of emotional behaviors in mice; and the functional neuroanatomy of innate behaviors in *Drosophila*.

Neural stem cell biology

Stem cells are multipotent, self-renewing progenitor cells. In the central nervous system (CNS), neurons, astrocytes and oligodendrocytes - the three major cell classes of the CNS are thought to be derived from self-renewing stem cells. In contradistinction to this prevailing view, our recent studies in the spinal cord suggest that following the period of neurogenesis, the majority of progenitor cells do not self-renew, but rather lose neurogenic capacity and become restricted to a glial fate. Current work is aimed at identifying genes that control this irreversible switch from neurogenesis to gliogenesis. An understanding of this mechanism might eventually permit controlled reversion of adult glial cells to a stem cell-like state, which could be useful for therapeutic purposes.

Neural circuitry of behavior

We are developing and applying molecular biological tools to map and manipulate the neural circuitry underlying emotional behaviors in mice. These studies focus on two distinct but related states: pain and fear. In the former case, we have identified a novel family of G protein-coupled receptors (GPCRs) for neuropeptides, called Mrgs, which are specifically expressed in restricted subsets of primary nociceptive sensory neurons. Using homologous recombination in embryonic stem cells, we have marked the neurons that express different Mrgs with genetically encoded axonal tracers. Remarkably, different Mrg-expressing neurons project to different, and highly specific, peripheral target tissues. We are now engaged in genetic inactivation or killing of these neurons, as well as genetic activation, to understand their function. We are also tracing the higher-order projections of these neurons into the brain, to determine the point at which these novel and distinct sensory circuits converge. In a separate project, we have identified genes that are expressed in subpopulations of neurons in the central nucleus of the amygdala, which is thought to be a major output structure that co-ordinates different aspects of learned fear responses. Using the promoters of these genes, we are generating mice in which these neurons can be reversibly silenced, in collaboration with Henry Lester's laboratory. The role of these neurons, and the circuits in which they participate, in mediating responses to conditioned and unconditioned fearful stimuli, can then be assessed.

In parallel with these studies in mice, we are engaged in conceptually similar experiments in the fruitfly, *Drosophila melanogaster*. Our goal is to identify simple and robust innate behaviors, and then perform unbiased "anatomical" and genetic screens to map the neuronal circuits and identify the genes that control these behaviors. This dual approach will provide an opportunity to integrate molecular genetic and circuit-level approaches to understanding how genes influence behavior. The "anatomical" screen exploits the availability of "enhancer trap" lines, in which the yeast transactivator protein GAL4 is expressed in specific subsets of neurons, and a conditional (temperature-sensitive) neuronal silencer gene (shiberets) that prevents synaptic transmission. Currently, we have developed assays for an innate avoidance response triggered by an odorant mixture released from traumatized flies, as well as for arousal intensity and hedonic valence, two important axes underlying emotional states in humans.

Selected Publications

Neural Development/Stem Cells

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C.J., Gronostajski, R.M. and Anderson, D.J. (2006) The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52**:953-968.

Mukouyama, Y.-S., Deneen, B., Lukaszewicz, A., Novitch, B.G., Wichterle, H., Jessell, T.M. and Anderson, D.J. (2006) Olig2+ neuroepithelial motoneuron progenitors are not multipotent stem cells *in vivo*. *Proc. Natl. Acad. Sci. USA* **103**:151-1556.

Pain/Nociception

Dong, X., Han, S., Zylka, M.J., Simon, M.I. and Anderson, D.J. (2001) A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons. *Cell* **106**:619-632.

Liu, Q., Vrontou, S., Rice, F.L., Zylka, M.J., Dong, X. and Anderson, D.J. (2007) Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. *Nature Neurosci.* **10**:946-948.

Zylka, M.J., Rice, F.L. and Anderson, D.J. (2005) Topographically distinct epidermal nociceptive circuits revealed by axonal tracers targeted to Mrgprd. *Neuron* **45**:17-25.

Fear/Amygdala

Choi, G.B., Dong, H.W., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., Swanson, L.W. and Anderson, D.J. (2005) Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus. *Neuron* **46**:647-660.

Han, C.J., O'Tuathaigh, C.M., van Trigt, L., Quinn, J.J., Fanselow, M.S., Mongeau, R., Koch, C. and Anderson, D.J. (2003) Trace but not delay fear conditioning requires attention and the anterior cingulate cortex. *Proc. Natl. Acad. Sci. USA* **100**:13087-13092.

Lerchner, W., Xiao, C., Nashmi, R., Slimko, E.M., van Trigt, L., Lester, H.A. and Anderson, D.J. (2007) Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated Cl-channel. *Neuron* **54**:35-49.

Mongeau, R., Miller, G.A., Chiang, E. and Anderson, D.J. (2003) Neural correlates of competing fear behaviors evoked by an innately aversive stimulus. *J. Neurosci.* **23**:3855-3868.

Zirlinger, M. and Anderson, D.J. (2003) Molecular dissection of the amygdala and its relevance to autism. *Genes Brain Behav*. **2**:282-294.

Fly Behavior

Carvalho, G.B., Kapahi, P., Anderson, D.J. and Benzer, S. (2006) Allocrine modulation of feeding behavior by the sex peptide of *Drosophila*. *Curr. Biol.* **16**:692-696.

Suh, G.S., Ben-Tabou de Leon, S., Tanimoto, H., Fiala, A., Benzer, S. and Anderson, D.J. (2007) Light activation of an innate olfactory avoidance response in *Drosophila*. *Curr. Biol.* **17**:905-908.

Suh, G.S., Wong, A.M., Hergarden, A.C., Wang, J.W., Simon, A.F., Benzer, S., Axel, R. and Anderson, D.J. (2004) A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* **431**:854-859.

207. A homeodomain code determines astrocyte positional identity in the spinal cord

Christian Hochstim, Benjamin Deneen, Qiao Zhou[*]*, David J. Anderson*

Astrocytes constitute the most abundant cell type in the CNS, and play diverse functional roles, suggesting phenotypic and ontogenetic cellular diversity. Positional identity is a fundamental organizing principle governing the generation of neuronal subtype diversity in the CNS, but whether it is relevant to astrocyte diversity is not clear. We have identified three positionally distinct subtypes of white matter astrocytes in the spinal cord, using the guidance molecules Reelin and Slit1 as molecular markers. We defined these ventral astrocyte subtypes as VA1 (Reelin+/Slit1-, located in the dorso-lateral white matter), VA2 (Reelin+/Slit1+, located in the ventro-lateral white matter), and VA3 (Slit1+/Reelin-, located in the ventro-medial white matter. Loss- and gain-of-function experiments indicate that the development of these astrocyte subtypes is controlled by homeodomain transcription factors, such as Pax6 and Nkx6.1, which at earlier stages determine positionally distinct subtypes of neurons. Thus, positional identity is an organizing principle underlying astrocyte diversification, and is controlled by a combinatorial homeodomain code whose elements are re-utilized following the specification of neuronal identity.

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208. Neural circuits responsible for *Drosophila* aggressive behavior

Kiichi Watanabe, David J. Anderson

Aggressive behavior is one of the important behaviors for animal survival and reproduction, throughout the animal kingdom from insects to humans. With powerful molecular and genetic tools, drosophila will provide great opportunities for study of molecular and circuitry mechanisms of aggressive behavior. However, because of its complexity, the neural circuitry or molecules responsible for this behavior are still unclear. Thus, we firstly have been developing automated methods to detect the specific behaviors that are observed in aggression, in collaboration with Dr. Heiko Dankert in Pietro Perona's lab (Electrical Engineering, Caltech). Until now, we have developed a computer-based fly tracking system to detect "lunging" behaviors, which is one of the typical aggressive behavior. Based on this system, I have started following projects:

a. Large-scale circuitry-based screening

As mentioned above, *Drosophila* has powerful molecular and genetic tools. Among them, Gal4-UAS system is one of the most commonly used techniques. In *Drosophila*, neuronal silencing by expression of potassium channels, such as Kir2.1 has been useful. To identify the neurons that are responsible for aggressive behavior, we have expressed Kir2.1 in specific neurons using various kinds of Gal4 lines and tried to see the phenotype on aggression behavior.

b. The role of octopamine in aggressive behavior

Octopamine, a biogenic monoamine structurally related to noradrenaline, acts as a neuromodulator in *Drosophila*,

and implicated in aggressive behavior. Although there are some reports relating to the roles of octopamine on aggressive behavior, it is still unclear which specific subsets of octopamine-synthesizing neurons or which octopamine receptors are involved in aggressive behavior. Firstly, we have tried to perform the knockdown analysis of each octopamine receptor using relevant UAS-RNAi constructs and pan-neuronal drivers such as Elav-Gal4 and Appl-Gal4. If we can identify the specific subsets of octopamine receptor that is responsible for aggression, we will generate their Gal4 driver lines and manipulate the activity of such neurons.

Thus, we have been trying to functionally dissect *Drosophila* brains to understand how specific neural circuits control aggressive behavior.

209. Sensitization and modeling emotional responses in *Drosophila*

Tim L. Lebestky, David J. Anderson

Emotional behaviors in humans convey a positive or negative response to a stimulus, and this response is typically manifest in discrete, highly conspicuous ways, such as stereotyped facial expressions and graded changes in levels of arousal. Although fruit flies (*Drosophila melanogaster)* do not present the richness of human emotions in their behavior, they may share fundamental molecular similarities that could allow us to dissect the way that neural circuits function to provide graded responses in arousal, as measured both qualitatively and quantitatively. To this end, we are developing automated, high-resolution behavioral assays that will allow a reproducible characterization of behavioral responses to aversive stimuli for high-throughput genetic screens.

One such assay follows the startle effects on locomotion and escape behaviors in response to a series of air-puffs, delivered at regular intervals. We observe a reproducible escalation of locomotor activity and jump-response behaviors as a function of time and puff number. The initial results suggest that the animals show a robust and sustained increase in locomotor arousal upon receiving a train of stimuli, but a sustained response is not observed upon the presentation of a single stimulus. This phenomena is typically referred to as "sensitization" and is a form of non-associative learning.

Our interpretation of the results is that the presentation of the puff stimuli raises the animals' state of acute arousal, as manifest in a high, sustained locomotor response, a sensitized state. We have performed pilot genetic screens to isolate and characterize insertional mutants and potential neural circuits that mediate this startle behavior, and are currently sorting through putative mutations. Two of these promising mutants are alleles in a dopamine receptor and also an allele in the *Drosophila* homolog of the Tachykinin receptor.

Our acquisition of the locomotor data is dependent on a video-tracking program that was developed jointly between the Perona and Anderson labs. The first generation tracking program is capable of accurately following many flies simultaneously (over 100) and generates basic velocity and trajectory data. We are currently improving and extending the basic program to provide the orientation of the animals to each other and higher forms of interaction.

210. Mrgprd$^+$ nociceptive neurons and pain sensation

Liching Lo, David J. Anderson

Mrgprd is expressed in a subset of IB4$^+$, non-peptidergic, nociceptive neurons. *Mrgprd*$^+$-free nerve endings terminate exclusively in the stratum granulosum layer of the eniepidermis. In the dorsal horn, *Mrgprd*$^+$ and CGRP$^+$ neurons project to distinct lamina layer IIm and lamina I layer, respectively. It has been suggested that parallel or distinct pain pathways may arise from these two different classes of nociceptors. The peptidergic CGRP$^+$ neurons and non-peptidergic IB4$^+$ neuron differ in where they project in the brain. The peptidergic neurons project to the parabrachial nucleus and thalamus, while the non-peptidergic neurons project to the limbic regions. These two classes of nociceptors have also been thought to mediate different adaptive pain pathways (inflammatory versus neuropathic). It is therefore important to genetically ablate or silence these neurons to reveal whether they have different sensory functions *in vivo*. We have made knock-in mice with a diphtheria toxin receptor (DTR, also known as human heparin-binding EGF like protein) targeted to the *Mrgprd* locus (D-DTR mice). Mice or rats are insensitive to diphtheria toxin (DTX) due to the fact that their surface receptors do not recognize the B portion of the DTX. Because of this, only *Mrgprd*-positive neurons which specifically express the DTR will be deleted by DTX. When we treated the D-DTR mice with DTX, nearly all the *Mrgprd* neurons lost the expression of DTR two days after injection. The central projection in the dorsal lamina IIm layer was also eliminated. Furthermore, the peripheral innervation in the epidermis was also missing. Taken together, we have shown successfully the acute ablation of a subset of IB4 sensory neurons. We are currently analyzing changes in pain behaviors in these mice. Because of the exclusive projection and innervation, it is important to know how the brain processes these specific afferent inputs and how this dedicated projection compares to other projections with different target specificities. Tracing the second or third order projections should provide us some answers. To achieve this goal, we are developing a human herpes virus that can be used for anterograde tracing from selected neurons. The virus encodes a GFP marker and replicates only in neurons that express Cre recombinase and the subsequent neurons that are in contact with the originally infected neurons postsynaptically.

211. Automated monitoring and analysis of the complex behavior of fruit flies

Heiko Dankert, David J. Anderson

Two different hardware setups and various algorithms for the automated monitoring and analysis of fruit fly behaviors have been developed. The first setup consists of a single chamber for the detailed monitoring and analysis of behaviors and social interactions between two flies. The second setup has five arenas and allows a high throughput screening.

As a basis for our analysis a fly detection and tracking software is developed. It extracts accurate and detailed information on appearance, shape, and movements of flies. Using these features, software detects fly behaviors such as wing extension, chasing, and circling - individually for each fly. An automated method for extracting bout structure data from simple locomotor behavior of populations of moving flies is under development.

A novel method for automated analysis and recognition of complex behaviors such as the aggressive lunging and tussling behaviors as well as courtship, is under development. Aggression is more variable in the laboratory, but less studied. Courtship is a more robust and reproducible behavior, but more studied. It recruits a wide variety of genes for its realization, including those concerning sex determination, ion channels, and circadian rhythms. These behaviors are usually manually scored, which is both laborious and only semi-quantitative. The new method is already in use for studying the lunging behavior of aggressive mutants of *Drosophila*. It allows quantifying and analyzing the effects of genetic and neural circuit-level manipulations on aggressive behavior, which represents an enormous step forward.

A second novel approach is the fully unsupervised discrimination between behaviors and detection of new behaviors. The method is further able to analyze pairs of mutant flies regarding their behavior and predict their mutation. It will provide important new insights into the genetic and neural control of behavior; on a level not previously achieved. The method allows us, for the first time, to determine the effect of social interactions on their locomotor behavior.

212. Control of neural stem-to-progenitor transition by CyclinD family members

Agnes Lukaszewicz

Embryonic stem (hES) cells are believed to soon be a key tool to repair or replace diseased or damaged tissue. For instance, replacing motorneurons (MN) degenerating in Amyotrophic Lateral Sclerosis appears to be one of the most promising treatments for this disease. Improving the knowledge of molecular mechanisms regulating the differentiation of ES cells into neurons is the first crucial step to achieve this goal.

Dr. David Anderson's lab has been interested for several years in understanding the molecular control of MN fate specification during development. MNs are derived from a specific progenitor domain: the pMN domain of the spinal cord. While carrying out a systematic characterization of changes in gene expression in this domain, genes coding for cell cycle regulators, the CyclinDs, have been isolated as potential candidates to regulate the stem cell homeostasis in the developing spinal cord.

CyclinD1 and D2 have been identified as specifically expressed in distinct subsets of precursors, and dynamically regulated during the neuronal to glia transition. This led us to hypothesize that CyclinD1 may regulate neurogenesis, whereas CyclinD2 may regulate the maintenance of the NSC. By modulating CyclinD1 levels of expression, we observed effects on neurogenesis consistent with our hypothesis. Furthermore, we showed that CyclinD1 re-expression is sufficient for glial-restricted progenitors to regain their neurogenic potential when transplanted into a permissive environment. Hence, we demonstrated that CyclinD1, independently of any effects on the cell cycle, modulates the generation of MN. We believe this constitutes an unexpected result of great importance for the field.

In parallel, CyclinD2 seems to exert opposite effects on neurogenesis. In order to determine if this reflects a role in maintaining NSC in the developing spinal cord, the impact of CyclinD2 expression on neurosphere formation activity is now being tested. Furthermore, these exciting observations raise the question whether the modulation of CyclinD1/D2 level of expression could be a way to influence ES cells along the *in vitro* neuronal differentiation pathway. This possibility will be tested by modulating CyclinDs level of expression in differentiating ES cells.

213. The role of neurosecretory cells in the modulation of *Drosophila* behavior

Timothy Tayler, Anne Hergarden, David J. Anderson

Animals exhibit countless complex and stereotyped behaviors such as aggression, courtship and the fight or flight response. These behaviors are generated and modulated by neural circuits. Other than a few simple reflex circuits, relatively little is known about how these circuits generate appropriate behaviors. *Drosophila* exhibit complex behaviors, but are anatomically less complicated and genetically more tractable than many vertebrate model systems. In addition, flies have a highly developed set of molecular tools that can be used to manipulate and analyze specific cell populations. Additionally, previous studies have demonstrated that *Drosophila* can be used to successfully identify neural correlates underlying complex behaviors such as courtship, olfactory aversion and learning and memory.

The goal of this project is to elucidate the connectivity, function, and modulation of circuits that underlie *Drosophila* behavior. Neuropeptides are an important class of signaling molecules that are involved in various aspects of animal physiology and behavior. To gain genetic control over neuropeptide-producing neurons we have identified the putative regulatory regions of 17 neuropeptide genes and have generated transgenic animals that express the GAL4 protein in the same pattern as these neuropeptides. The Gal4/UAS system is a genetic tool that enables us to express a large variety of transgenes in a spatially- and temporally-controlled manner. We have used fluorescent reporters to characterize

these neuropeptide-Gal4 lines and have also validated the Gal4 expression patterns. We are currently using these newly generated tools to try to learn about the role of neuropeptidergic neurons in modulating behaviors. This is accomplished by using the neuropeptide-gal4 lines to express molecules that can either silence or activate neurons. We are now testing these circuit-modified animals in a wide variety of behavioral paradigms including feeding behavior, courtship, and circadian rhythm.

214. Neuronal control of locomotor activity in the fruitfly

Allan M. Wong, Michael H. Dickinson[*]*, David J. Anderson*

The fruitfly when presented with visual or olfactory stimuli responds with a change in behavior. For instance, when flies see a long thin strip in their visual field they will move towards the stripe; when exposed to low concentrations of carbon dioxide the fly will move away from the gas. In order to accomplish these behaviors, the fly must detect environmental changes, decide what to do, and coordinate its muscles to accomplish the behavior. We wish to understand how these steps are encoded by the nervous system of the fly. We reason a good entry point is to understand the connectivity and function of the descending interneurons. These neurons connect the brain with the thoracic ganglia (the equivalent of the spinal cord in vertebrates). All visual- and olfactory-mediated behaviors must be transmitted through these neurons, as they are perceived in the eyes and antenna in head and manifested through the wings and legs on the throax.

In the past year, we, along with Will Dickson and Andrew Straw, have adapted and improved on Erich Buchner's tethered walking fly arena in which a tethered fly is walking on top of a floating Styrofoam ball. This experimental setup simulates walking behavior while keeping the fly stationary, permitting tightly controlled stimulus-response experiments that can be coupled with functional imaging and electrophysiological recordings to monitor neural activity. We have integrated the tethered fly setup with the virtual flight arena hardware developed by Michael Riser to present visual stimuli to the walking fly. We also developed a preparation where we remove cuticle from the fly head to permit two-photon imaging in the fly. Importantly, flies with these surgical procedures can still respond to visual stimuli as recorded on the tethered walking arena.

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215. Connectivity and function of lateral septal Crhr2^{+} neurons

Todd Anthony, David J. Anderson

Although several brain regions have been implicated in regulating anxiety, the specific neural circuits involved remain poorly understood. The identification and analysis of these circuits is therefore a fundamental first step towards the development of improved treatments for anxiety disorders. The lateral septum (LS) is one brain region that has long been thought to be involved in controlling anxiety. However, it is unclear whether the LS is primarily anxiolytic or anxiogenic, or perhaps comprised of multiple classes of neurons each with a unique effect on anxiety. Answering this question will require systematically manipulating defined LS neuronal populations in a precise and reproducible manner. One particular population likely to be relevant to analysis of the LS role in anxiety are neurons that express the corticotropin-releasing hormone receptor 2 (Crhr2); genetic data has demonstrated that Crhr2 modulates behavioral responses to stress and anxiety, and pharmacological studies have shown that these effects are due at least in part to Crhr2-expressing neurons within the LS. However, despite strong evidence implicating LS Crhr2^{+} neurons in regulating anxiety, neither their synaptic connections nor exact function are known. We are addressing these questions using genetic tools to trace the connectivity and manipulate the firing properties of LS neurons that express Crhr2. By restricting analysis to neurons expressing Crhr2, these experiments will yield highly specific results about an anatomically- and genetically-defined neuronal population; such information is a prerequisite in the development of targeted anxiolytic drugs.

216. Differentiation of reproductive and aggression circuits in rodents

Dayu Lin, David J. Anderson

Inter-male aggression and male-female mating are generally considered as distinct and mutually incompatible behaviors. Nevertheless, it is increasingly recognized that these opponent behaviors may share similar neurobiological and neuroendocrine mechanisms. For example, both mating and territorial aggression are dependent upon circulating gonadal steroids such as testosterone and estradiol. Both behaviors are accompanied by a high arousal state, reflected by increased heart rate and elevated blood pressure. Moreover, both behaviors rely heavily on olfactory and pheromonal input. Ablation of either the main olfactory epithelium (MOE) or vomeronasal organ (VNO) diminishes both mating and agonistic behaviors. Lesions of the medial hypothalamus and medial amygdala in rodents decrease the occurrences of both mating and fighting. Mapping of neuronal activation using c-fos induction in hamsters revealed largely overlapping patterns associated with these two behaviors. Taken together, these data suggest that mating and agonistic behaviors may be subserved by a common network of steroid hormone sensitive limbic circuits. It is unclear how these two heavily overlapping pathways produce two opposite behavioral outcomes. One possibility is that two intermingled but distinct subpopulations of neurons in the same regions mediate mating and fighting. Alternatively, the same population of neurons mediates both mating and fighting through neuromodulation. The goal of the current study is to distinguish these two signal-processing scenarios. We first performed between-animal comparisons of the patterns of brain activation during mating and fighting, using c-fos

analytic methods that permits rapid sampling across the entire brain. Our preliminary results indicate that mating and fighting indeed activate many similar hypothalamic and amygdalar regions in mice. However these areas are distinct from those activated during anti-predator defense. To further understand whether the same population of neurons mediates both behaviors, we are adapting a method to compare c-fos expression induced during the two behaviors in the same animal. Our preliminary data suggest that, at least in some commonly activated regions, two distinctive sets of neurons are likely involved in mating and fighting.

217. Investigation of behavior and neural circuitry underlying mechanosensation in *Drosophila melanogaster*

Suzuko Yorozu, David J. Anderson

Mechanosensation is intricately linked to an organism's survival. For example, the ability to detect wind allows many flying insects to navigate, while other insects detect disturbances of air particles to help them locate predators and prey. Thus, a coordinated locomotor output in response to mechanical stimuli is critical for survival of many insects. The link between mechanosensation and locomotor control at the neural circuit level is currently not well understood. In my project, I am trying to understand the fly's behavioral and neuronal responses to wind stimulation using a novel behavioral assay called Air Suppression of Locomotion (ASL). In the ASL assay, wild-type adult flies immediately slow their locomotor activity in response to wind stimulation. They remain immobile for as long as they are exposed to wind stimulation, while they immediately re-engage in locomotor activity when the wind stops, as if the wind stimulus acts as a neural switch for fly's locomotor activity. Interestingly, when the third antennal segment is surgically removed bilaterally, the flies continue to engage in locomotor activity in the presence of air flow stimulation, suggesting that detection of air current is probably mediated through sensory organs housed in the antennae. We propose to take an advantage of the simplicity, robustness, high reproducibility, and unique locomotor phenotype of ASL to gain insight into the link between mechanosensation and locomotor control, at the neural circuit level. The goal of my project is to understand where and how air currents act on the nervous system to suppress locomotor activity in the fly. To this end, we will combine behavioral genetics, electrophysiology and calcium response imaging to investigate: 1) which sensory organ mediates ASL; and 2) identify the second- and higher-order neurons that mediate the processing of wind detection and suppression of locomotor activity.

218. The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord

Ben Deneen, David J. Anderson

The mechanisms controlling the transition from neurogenesis to giliogenesis in the vertebrate CNS are incompletely understood. We identified a family of transcription factors, called NFI genes, which are induced throughout the spinal cord ventricular zone (VZ) concomitantly with the induction of GLAST, an early marker of gliogenesis. NFIA is both necessary and sufficient for GLAST induction in the VZ. Unexpectedly, NFIA is also essential for the continued inhibition of neurogenesis in VZ progenitors. This function is mediated by the requirement of NFIA for the expression of HES5, a Notch effector. However Notch effectors are unable to promote glial fate specification in the absence of NFIA. Thus, NFIA links the abrogation of neurogenesis to a generic program of gliogenesis, in both astrocyte and oligodendrocyte VZ progenitors. At later stages, NFIA promotes migration and differentiation of astrocyte precursors, a function that is antagonized in oligodendrocyte precursors by Olig2.

219. Modulation of fear behaviors in the central amygdala

Wulf Haubensak, David J. Anderson

Fear is probably the most conserved emotion, underlying defensive behaviors across species, and, in turn, it is a basic, medically important, human emotion that can be addressed in experimentally tractable animal model systems. Numerous studies have pointed to a central role of medial temporal lobe structures, particularly the amygdala, in various forms of fear. However, the surgical and pharmacological methods used were too coarse to study single neuronal circuits with cellular resolution. This is especially important when investigating how fear stimuli are processed in the different amygdala subregions, and the different neuronal populations therein. Among these, neurons in the central nucleus (CeA), which relays signals to lower brain regions activating the release of stress hormones, autonomic and motor responses, can be expected to play a central role in fear processing. Here, we explore a genetic strategy in mice to analyze function and circuitry of neurons with higher resolution.

We have selected PKC-delta, which is strongly expressed in the CeA, as a region-specific gene for genetic targeting of CeA neurons. However, as for many other genes, PKC-delta is expressed in more than one brain region. We therefore generated PKC-delta transgenic mice expressing one of the two subunits of a chloride channel for silencing (Slimko *et al., J. Neurosci.* **22**:7373), and delivered the second subunit by stereotaxic injection of Adeno-associated virus into the CeA, reconstituting the functional channel only in PKC-delta neurons.

This approach has allowed us to selectively silence PKC-delta cells in a temporally defined manner, and to investigate their function in fear behaviors. Preliminary results suggest that these neurons may be part of a local circuit in the CeA that integrates stressful and stress coping signals. We now aim to corroborate this by stereotactic injections of Cre-dependent viruses for neuronal tracing into the CeA of a PKC-delta::Cre transgenic mouse line to identify projections to and from these neurons.

220. Molecular genetic and physiological analyses of neural circuits underlying innate behavior in *Drosophila*

Greg S. Suh

Animals in the wild encounter many types of external stimuli such as threat and food, and must exhibit appropriate responses for survival. How does a brain recognize such stimuli with sensory systems, create internal representations of these external stimuli, and then elicit appropriate behavioral responses? To address this problem, I have been taking a systems approach and using the fruit fly, *Drosophila melanogaster*, because of a wealth of genetic tools available, a relatively simple brain, and a complex, interesting behavioral repertoire. Rapidly emerging tools also permit relatively facile identification of neural substrates. My focus has been to identify neurons that subserve a particular innate behavior, apply functional imaging and electrophysiology to probe their activity, and therefore define precisely contributions of each set of neurons to the behavior.

We have developed a novel behavioral paradigm in *Drosophila* in collaboration with Seymour Benzer. This paradigm involves avoidance of a substance, called *Drosophila* Stress Odorant (or dSO) emitted by flies subjected to mechanical stress or electrical shock. Most naive flies chose the fresh tube when given a choice in a T-maze between a fresh tube and a conditioned tube in which emitter flies were previously stressed. Through Gas Chromatography & Mass Spectometry (GC/MS) analyses, I identified CO_2 as one component of dSO. Consistent with this, flies exhibited avoidance to CO_2 in a dosage-dependent manner. We next identified a single pair of glomeruli in the antenna lobe (AL) innervated by Gr21a+-expressing olfactory neurons that were specifically activated by CO_2 through functional imaging. And this paired glomeruli (the V glomeruli) were necessary for avoidance to CO_2. Furthermore, activation of Gr21a+-expressing neurons using recently developed Channelrhodopsin-2 elicited avoidance response to its stimulus, blue light. These experiments together indicate that Gr21a+-expressing neurons are dedicated to detecting CO_2, and that avoidance to CO_2 is likely mediated by a dedicated circuit. It is worth emphasizing that the CO_2 neuron is another rare example of an "object detector" found in the peripheral nervous system.

References

Suh, G.S., Ben-Tabou de Leon, S., Tanimoto, H., Fiala, A., Benzer, S. and Anderson, D.J. (2007) *Curr. Biol.* **17**(10):905-908.

Suh, G.S., Wong, A.M., Hergarden, A.C., Wang, J.W., Simon, A.F., Benzer, S., Axel, R. and Anderson, D.J. (2004) *Nature* **431**:854-859.

221. A common genetic target for environmental and heritable influences on aggressiveness in *Drosophila*

Liming Wang, David J. Anderson

Both genetic selection and social isolation can promote increased aggressiveness. Microarray analysis of socially-isolated vs. group-housed *Drosophila* identified Cyp6a20, a cytochrome P450, as the only differentially expressed gene in common with a set previously identified by profiling *Drosophila* strains selected for differential aggressiveness. Social isolation decreased Cyp6a20 expression, and increased aggressiveness, in a reversible manner. In Cyp6a20 mutants, the suppression of aggressiveness by group housing was diminished. These data identify a common genetic target for environmental and genetic influences on aggressiveness. Cyp6a20 is expressed in olfactory support cells, suggesting that social experience influences aggressiveness via regulation of olfaction.

Reference

Dierick, H.A. and Greenspan, R.J. (2006) *Nature Genet.* **38**:1023.

222. Genetic manipulation of neuronal subpopulations involved in pain and pleasure

Sophia Vrontou, David J. Anderson

Studies in our lab have identified a novel murine family of G-protein-coupled receptors (GPCRs), which is related to proto-oncogene MAS1 and called Mas-related genes (Mrgs). Mrgs As, Bs, C and D thus far analyzed, are specifically expressed in mostly non-overlapping subpopulations of trigeminal and dorsal root ganglion (DRG) small-diameter sensory neurons, implying that they might have a potential nociceptive role. Most surprisingly it was found that the expression of MrgD is restricted to exclusively cutaneous peripheral afferents, rendering MrgD the first specific molecular marker that predicts the end-organ specificity of a subset of primary sensory neurons, and supporting the existence of a molecularly distinct subpopulation of cutaneous nociceptors. These data raise the question of: a) whether there are other molecularly distinct nociceptor subsets, innervating different targets and, b) whether the circuits they engage in follow separate pathways and up to what point they might intersect into the brain. We are looking for molecular markers for such subsets and especially for visceral nociceptors, since visceral pain is the most common but still understudied form of pain. We will use these markers to compare the circuitry of their expressing neurons with that of nociceptor subsets innervating other targets, such as the skin and also to genetically manipulate them so as to identify their function.

Most recently, anatomical analysis of MrgB4-expressing neurons revealed that these neurons constitute a rare population of small–diameter sensory neurons, innervating exclusively the hairy skin. It is also suggested that they might mark the mice analogs of the so-called C-fiber tactile (CT) afferents in humans that respond to gentle stroking. We are interested in deciphering the anatomy of this specific subpopulation in the upper brain regions and

most importantly in identifying their function by measurement of their activity *in vivo* using gene targeting technology.

Publications

Deneen, B., Lukaszewicz, A., Hochstim, C.J., Gronostajski, R.M. and Anderson, D.J. (2006) The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52:**953-968.

Lerchner, W., Xiao C., Nashmi R., Slimko, E.M., Van Trigt, L., Lester, H.A. and Anderson, D.J. (2007) Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated cl(-) channel. *Neuron* **54**(1):35-49.

Liu, Q., Vrontou, S., Rice, F.L., Zylka, M.J., Dong, X. and Anderson, D.J. (2007) Molecular genetic visualization of a rare subset of unmyelinated sensory neurons that may detect gentle touch. *Nature Neurosci.* **10**:946-948.

Rebelo, S., Chen, Z.F., Anderson, D.J. and Lima, D. (2006) Involvement of DRG11 in the development of the primary afferent nociceptive system. *Mol. Cell Neurosci.* **33**:236-246.

Suh, G.S.B., Ben-Tabou de leon, S., Tanimoto, H., Fiala, A., Benzer, S. and Anderson, D.J. (2007) Light-activation of an innate olfactory avoidance response in *Drosophila*. *Curr. Biol.* **17**:905-908.

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Postdoctoral Fellows: Bader Al-Anzi, Shlomo Ben-Tabou de Leon, William Ja., David W. Walker
Research and Laboratory Staff: Stephanie Cornelison, Noelle De La Rosa, Nick Lawrence, Viveca Sapin, John Silverlake, Rosalind Young
Graduate Students: Gil Carvalho, Julien Muffat, Brian Zid

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Summary: Our group uses *Drosophila* as a model system in which to identify and characterize genes involved in behavior, aging, and neurodegeneration. The high degree of homology between the fly and human genomes forms the basis of a strategy for understanding the corresponding human genes.

223. A *Drosophila* model of Leigh Syndrome

David W. Walker, Seymour Benzer

Mutations in mitochondrial genes, encoded by mitochondrial DNA or nuclear DNA, have been implicated in a wide range of neuromuscular diseases. Complex I (NADH dehydrogenase) deficiency is the most common enzyme defect among mitochondrial disorders (estimated incidence of 1 in 10,000 live births). Leigh syndrome, an early-onset progressive neurodegenerative disorder, is associated with up to 50% of cases of complex I deficiency. Although patient studies have revealed much with regard to the genetics of mitochondrial disease, the pathophysiology of these diseases remains poorly understood. What is needed is the development of animal models for these diseases that are amenable to detailed biochemical, molecular and physiological analysis.

We are using *Drosophila* genetics to dissect pathways of disease pathogenesis resulting from mitochondrial dysfunction. Previously, we have isolated and characterized a fly mutant with a defect in mitochondrial respiratory complex II. We have started to perform a similar analysis of a fly mutant carrying a defect in respiratory complex I. The affected gene is the ortholog of a human gene, NDUFV1, mutations in which cause Leigh syndrome. The fly gene, CG9140, shares 88% amino acid homology with NDUFV1 and is located on the left arm of the second chromosome. We are using targeted RNAi inactivation to inhibit the expression of CG9140 in different tissues of the fly. RNAi inactivation of CG9140 within the nervous system recapitulates the major features of Leigh syndrome: early death, neurodegeneration, motor defects and mitochondrial ultrastructural changes. The next step will be to examine the physiological consequences of complex I deficiency that cause this syndrome, and then to harness the power of *Drosophila* genetics to identify possible targets for therapeutic intervention.

224. Effect of mitochondrial dysfunction mutation on egg laying behavior in *Drosophila*

Shlomo Ben-Tabou de-Leon, Gil Carvalho, Seymour Benzer

Reactive oxygen species (ROS) have been shown to play an important role in many signaling pathways, and mutations that change the ROS homeostasis can affect cellular processes that depend on ROS-mediated signaling. In *C. elegans*, for example, it was shown that a mutation that reduces ROS levels causes delayed maturation of the gonads and delayed onset of egg production, by affecting two different signaling pathways. Both these phenotypes were rescued by reducing the activity of the cytoplasmic enzyme superoxide dismutase (SOD) thus elevating the ROS level.

A *Drosophila sdhB* mutant identified and characterized in our lab by David Walker shows impaired mitochondrial complex II-mediated electron transport that is associated with an increased level of hydrogen peroxide (H_2O_2) production. We have found that this mutant has altered egg-laying behavior: virgin *sdhB* females start to lay eggs much earlier than do control flies (1 day vs. 4-5 days after eclosion) and thereafter continue at a higher egg-laying rate. To investigate the possibility that elevated ROS level in the *sdhB* mutant might account for the difference, we measured the egg-laying rate in wild-type (Canton-S) virgin females that were exposed to hyperoxia (100% O_2). In hyperoxic conditions, virgin CS females showed increased egg laying rate, compared to control flies in normoxia, suggesting that, in the *sdhB* mutant, the early onset and increased rate of egg laying might be the result of its increased ROS level.

225. 4EBP modulates lifespan and mitochondrial function upon dietary restriction in *Drosophila*

Brian M. Zid, Pankaj Kapahi, Seymour Benzer

Dietary restriction (DR) extends lifespan in various organisms, yet its molecular underpinnings are poorly understood. Previously, we and other labs have found that the TOR (target of rapamycin) pathway, a conserved nutrient-sensing growth pathway, is a determinant of lifespan that interacts with DR in multiple organisms. To further understand the molecular mechanisms of DR, we focused on the eukaryotic translation initiation factor 4E binding protein, 4EBP, a downstream mediator of the TOR pathway in *Drosophila*. We found that DR upregulates 4EBP, and that this upregulation is necessary and sufficient for the full lifespan extension upon DR. Given the role of 4EBP in regulating the translation initiation factor eIF4E, we

investigated the genome-wide translational changes occurring under DR, using translation state array analysis (TSAA). Categories enriched among the translationally downregulated genes were those for carboxylic acid, cellular biosynthesis and carbohydrate metabolism, while the increased translation gene class was enriched for mitochondrial ATP generation, oxidative phosphorylation and protein folding. Upregulation of mitochondrial function was verified and found to be *d4EBP* dependent, suggesting a novel mechanism for regulating mitochondrial function upon DR. These results demonstrate a key role for mRNA translation initiation in modulating lifespan and mitochondrial function upon DR.

226. Modeling the role of Apolipoprotein D in longevity and stress resistance, in *Drosophila*

Julien A. Muffat, David W. Walker, Seymour Benzer

We have been studying the roles of Apolipoprotein D and its fly homologs *Glial Lazarillo (Glaz)* and *Neural Lazarillo (Nlaz).* ApoD is a soluble lipoprotein thought to bind small hydrophobic molecules, such as arachidonic acid and sterols, perhaps capable of clearing away damaging molecules, or rather providing building blocks for cell membranes. Remarkably, it accumulates at the site of many neurodegenerative lesions, but its physiological function in such pathological situations is unknown. We are using the strengths of *Drosophila* genetics to manipulate the levels, location, and timing of expression of the fly homologs, as well as human ApoD.

We previously reported that overexpression of *Glaz* is sufficient to cause life extension and increased multiple stress resistance in *Drosophila*. We have now extended these findings to the other fly homolog, *Nlaz*. Flies carrying *Nlaz* RNAi are sensitive to oxidative stress and short-lived under normal conditions, while *Nlaz* overexpressors are, conversely, long-lived and stress resistant. By study of the transcriptional regulation of *Glaz* and *Nlaz* expression with real-time quantitative PCR, we found that *Glaz* is very strongly upregulated within hours of exposure to various stressors, including pure oxygen and heat. *Nlaz* is also upregulated, albeit to a lesser extent. We generated transgenic flies overexpressing human ApoD, and found them to be 40% longer-lived than wild-type flies, and resistant to oxidative stress, providing the first model of human ApoD overexpression, and supporting the beneficial role of human ApoD upregulation previously indicated by the fly homolog data.

Reactive oxygen species (ROS) are thought to play an important role in both normal and pathological aging. Among the end products of oxidative stress from ROS are lipid peroxides in cell membranes, which disrupt normal membrane physiology. We have shown that flies overexpressing human ApoD accumulate far less lipid peroxides with age than do wild-type flies. This suggests a mechanism for the beneficial action of ApoD on aging or diseased tissues: it may bind and quench lipid peroxides, restore normal membrane physiology, or prevent their formation by scavenging free radicals. In a *Drosophila* cell culture system, we found that overexpression of Glaz and Nlaz are both capable of rescuing cell death triggered by exposure to paraquat, a model of Parkinson's disease, and beta-amyloid, the peptide responsible for Alzheimer's disease. Together, these data support a role of these lipoproteins as part of a natural stress response, conserved between flies and humans.

227. Physiological functions for Neural Lazarillo in *Drosophila*

Julien A. Muffat, David W. Walker, Seymour Benzer

In adult flies, *Glaz* is normally expressed in a subset of glial cells and hemocytes. In contrast, *Nlaz* expression, visualized using a GFP reporter construct, is restricted to sensory neurons with projections to the sub-esophageal ganglia. Gustatory neurons of the forelegs, as well as the ones on the labellum, appear to be the main inputs to that circuit. *NLaz* belongs to a superfamily of lipocalins that also includes - odorant-binding proteins. We hypothesize that its expression in this sensory network is related to a possible function as the carrier of molecules that can then be recognized by gustatory or olfactory receptors. A simple assay of gustatory response is the proboscis extension test: when a drop of sugar water is touched to the fly's forelegs, a reflex extension of the mouthparts occurs. This response can be modulated by appetizing or aversive substances. When we knock down *NLaz* ubiquitously, using an RNAi line driven in all tissues, we observe a significant drop in this response.

In larvae, our GFP reporter for *Nlaz* fails to show neuronal expression, but seems to localize to the anterior spiracle gland, one of the active sites of cuticular hydrocarbon secretion for the larva's exoskeleton and tracheal network. The exoskeleton is very hydrophobic, due to the presence of many lipidic molecules. In is conceivable that *NLaz*, and perhaps *GLaz*, may bind to and transport some of these cuticular hydrocarbons. Interestingly, one of the overexpression phenotypes is resistance to dry starvation, which could be related to permeability of the cuticle.

228. *Drosophila* appetite and prandiology studied with the CAFE assay

William W. Ja, Gil Carvalho, Elizabeth Mak, Brooke Hunter, Seymour Benzer

Studies of feeding behavior in genetically tractable invertebrate model systems have been limited by the lack of proper methodology. We developed the Capillary Feeder (CAFE), a method allowing precise, real-time measurement of ingestion by individual or grouped fruit flies, on the scale of minutes to days. Using this technique, we conducted the first quantitative analysis of prandial behavior in *Drosophila melanogaster*. Our results allow the dissection of feeding into discrete bouts of ingestion, defining two separate parameters, meal volume and frequency, that can be uncoupled, and thus are likely

to be independently regulated. We used the CAFE to test the effects of dietary supplementation with paraquat or ethanol on food ingestion and preference. Paraquat, a prooxidant widely used in stress tests, had a strong anorexigenic effect. In contrast, in feeding preference assays, ethanol-laced food, but not ethanol by itself, acted as an attractant.

Previous work in our laboratory suggests that capsaicin can affect food preference after a period of starvation. This observation is surprising since, in the fly, capsaicin does not elicit a detectable activation of gustatory neurons. Using the CAFE, we confirmed that flies prefer a capsaicin-laced sugar solution to sugar alone when starved, but not when satiated. Strikingly, this effect is seen when flies are housed in groups, but not when kept in isolation, suggesting a phenomenon of social facilitation. We are currently investigating the mechanisms underlying this behavior.

Using a collection of lines expressing double-stranded RNAi targeted against various *Drosophila* genes, we are conducting a screen for mutants showing abnormal food intake. We have isolated various lines showing reduced appetite, and a mutant with a 50% increase in food ingestion. We are currently characterizing the behavior of these mutants on a range of foods of increasing nutrient concentration, as well as analyzing metabolic parameters such as fat content and motor activity. In addition, we are characterizing the prandial behavior of the mutant strains. For this purpose, we have adapted the CAFE to automatically collect high-resolution recordings. Flies housed individually in small chambers are monitored using a digital camera programmed to record images every 5 min for a period of several hours. The pictures collected are then processed using image-analysis software.

229. Odor and taste sensitivity, and habituation behavior, of *Drosophila dNOS* mutants

Victoria Bone, Angela Chang, Bader Al-Anzi, Seymour Benzer

Nitric oxide (NO) plays many important roles in biological processes, including regulation of blood flow and pressure, ion channel conductance, glycogenolysis, apoptosis, and smooth muscle relaxation. Defects in NO signaling can result in diseases such as stroke, heart disease, gastrointestinal distress, erectile dysfunction, and neurodegeneration. No is largely produced by the enzymatic activity of nitric oxide synthetase (NOS), an essential enzyme in most animals, and mediates the production of cyclic guanosine monophosphate (cGMP), which serves as a second messenger. In *Drosophila melanogaster* NOS is encoded by a single gene designated *dNOS*.

We have isolated P-element mutations in *dNOS* called $dNOS^{f24691}$, $dNOS^{c01670}$, and $dNOS^{e02671}$. Using an NO-specific stain, we observed, in the olfactory organs of normal adult flies, namely the antennae and maxillary palps, a high level of NO. This is markedly reduced in the mutants. Using a T-maze olfactory assay, we discovered that *dNOS* mutants are more sensitive to odors than wild-type controls, due largely to a defect in adaptation.

Through antibody staining, we also observe a high level of dNOS protein in the gustatory organs of normal flies, specifically in the labial palps and at the tips of the legs. This suggests possible involvement of *dNOS* in the fly's gustatory behavior, which we are currently investigating.

230. Light activation of an innate olfactory avoidance response in *Drosophila*

Greg S. B. Suh, Shlomo Ben-Tabou de Leon, Hiromu Tanimoto, Andrea Fiala, Seymour Benzer, David Anderson

How specific sensory stimuli evoke specific behaviors is a fundamental problem in neurobiology. In *Drosophila*, most odorants elicit attraction or avoidance, depending on their concentration, as well as their identity. Such odorants typically activate combinations of glomeruli in the antennal lobe of the brain, complicating the dissection of the circuits translating odor recognition into behavior. Carbon dioxide (CO_2), in contrast, elicits avoidance over a wide range of concentrations and activates only a single glomerulus, V. The V glomerulus receives projections from olfactory receptor neurons (ORNs) that coexpress two GPCRs, Gr21a and Gr63a, that together comprise a CO_2 receptor. These CO_2-sensitive ORNs are located in the ab1 sensilla of the antenna. Genetic silencing of ab1c neurons indicates that they are necessary for CO_2-avoidance behavior. Whether activation of these neurons alone is sufficient to elicit this behavior, or whether CO_2 avoidance requires additional inputs (e.g., from the respiratory system), is still unclear. We have shown that artificial stimulation of ab1c neurons with light (normally attractive to flies) elicits the avoidance behavior typical of CO_2. Thus, avoidance behavior appears hard-wired into the olfactory circuitry that detects CO_2 in *Drosophila*.

We expressed the photo-activated cation-selective channel rhodopsin-2 (ChR2) in ab1c neurons using a *Gr21a-Gal4* driver, and raised the flies either in food supplemented with all-*trans* retinal or, as a control, without the supplement. To determine whether light-activation of ChR2 can mimic the effect of CO_2 on ab1c activity, we recorded action potentials in a single ab1 sensillum. Indeed, 470 nm blue light elicited spike trains from ab1c neurons, but not from other ORNs in the sensillum. Two types of retinal-dependent spiking responses to light were seen in CO_2-responsive sensilla: a "non-adapting" response that persisted for the duration of the stimulus, and a transient response that terminated within ~200 msec following the stimulus onset. The persistent response was similar to that evoked by ~2% CO_2.

To determine whether light-activation of CO_2-sensitive ORNs is also sufficient to elicit avoidance behavior, a T-maze was constructed in which one arm was outfitted with water-cooled 470 nm light-emitting diodes. Flies were given 30-60 sec to choose, and then counted.

Flies expressing *Gr21a-Gal4* and two copies of *UAS-ChR2*, and raised on retinal-containing food, avoided the test arm when the LEDs were illuminated. Avoidance was not observed in flies expressing the driver nor the responder alone, nor in flies raised without retinal. This avoidance of blue light is in stark contrast to the normally strong attraction to blue light of wild-type flies. The demonstration that the activation of ab1c neurons by light is sufficient to elicit avoidance in a T-maze, taken together with the requirement of these neurons for CO_2 avoidance, indicates that this behavior is exclusively mediated by ab1c ORNs, and does not involve combinatorial or temporal coding of odor identity. It also indicates that CO_2 avoidance does not require other sensory input, e.g., from the respiratory system. The fact that activation of a single population of ORNs suffices to trigger avoidance further suggests that this behavior is hard-wired into the olfactory circuitry that detects CO_2 in *Drosophila*.

The use of ChR2 to activate neurons in adult *Drosophila* provides a valuable complement to other genetically based neuronal photo-activation techniques.

Publications

Ja, W.W., Carvalho, G.B., Mak, E.M., de la Rosa, N.N., Fang, A.Y., Liong, J.C., Brummel, T. and Benzer, S. (2007) Prandiology of *Drosophila* and the CAFE assay. *Proc. Natl. Acad. Sci. USA* **104**:8253-8256.

Ja, W.W., West, Jr., A.P., Delker, S.L., Bjorkman, P.J., Benzer, S. and Roberts, R.W. (2007) Extension of *Drosophila melanogaster* lifespan with a GPCR peptide inhibitor. *Nature Chem. Biol.* Published online June 3, 2007.

Suh, G.S.B., Ben-Tabou de Leon, S., Tanimoto, H., Fiala, A., Benzer, S. and Anderson, D.J. (2007) Light-activation of an innate olfactory avoidance response in *Drosophila*. *Curr. Biol*. **17**:905-908.

Lois and Victor Troendle Professor of Cognitive and Behavioral Biology, Professor of Biology and Engineering and Applied Science: Christof Koch
Research Fellows: Costas Anastassiou, Wolfgang Einhäuser, Ila Fiete, Masaki Fukuchi, Prashant Joshi, Sotiris Masmanidis, Josh Milstein, Florian Mormann, Vidhya Navalpakkam, Melissa Saenz, Claudia Wilimzig
Graduate Students: Moran Cerf, Dan Cleary, Will Coulter, Julien Dubois, Carl Gold, Jonathan Harel, Andreas Kotowicz, Farshad Moradi, Kerstin Preuschoff, Ueli Rutishauser, Nao Tsuchiya, Dirk Walther, Stephen Waydo
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Summary: Research in the laboratory focuses on three areas: (1) biophysics of computation in nerve cells; (2) understanding visual selective attention and visual consciousness at the neuronal, behavioral and computational levels; (3) based on this understanding, develop biological-motivated vision algorithm and apply them to the automatic recognition of objects. For more details and all publications, see http://www.klab.caltech.edu

Research carried out as part of a "Biophysics of Computation" focuses on how the electrophysiology, synaptic architecture, and dendritic morphology of groups of individual neurons subserve information processing. What are the biophysical mechanisms underlying neuronal computations? Can we infer something about the shape and property of a neuron by listening to its extracellular observed action potential? How does the constantly fluctuating extracellular membrane potential influence the intracellular membrane potential? Can the extracellular membrane potential carry information of relevance to neural computation? Analytical work, backed up by detailed computer simulations of nerve cells based on electrophysiological data from our experimental collaborators at the Hebrew University in Jerusalem (Idan Segev and Yosef Yarom) and generates experimentally verifiable predictions. With Guyri Buzsaki at Rutgers, we continue to investigate the relationship between extra- and intracellular spike waveforms and the genesis of the local field potential.

Our laboratory collaborates with the neurosurgeon and neuroscientist Itzhak Fried at UCLA, recording from 64 electrodes in the medial temporal lobe of awake patients with pharmacologically intractable epilepsy who are implanted with depth electrodes to localize the focus of seizure onset. This unique setting allows us to observe invariant recognition, imagery and representation of familiar objects and famous individuals in conscious humans by listening in on the spiking activity of many individual neurons - complemented by local field analysis. This work requires sophisticated data processing skills and the careful design of the appropriate behavioral-physiological paradigms that will work in a clinical context.

Understanding the action of selective, visual attention (both saliency-driven, bottom-up, as well as task-dependent, top-down forms) requires a firm grasp of how visual object recognition in natural scenes can be solved at the computational level, and how the resulting algorithms can be mapped onto the known architecture of the visual cortex and associated cortical and sub-cortical areas. We use analytical methods, coupled with computer simulations of the appropriate circuitry in the primate visual system, visual psychophysics, eye tracking and functional brain imaging at Caltech's 3.0 T Trio scanner to investigate human object recognition in the near-absence of focal attention, in visual search and in natural scene perception (some of this is done in collaboration with Laurent Itti at USC). Together with Tomaso Poggio at MIT, we investigate neurobiologically plausible models of both the ventral and dorsal visual streams. What are the limits of human object recognition? To what extent can probabilistic Bayesian recognition algorithms perform near-human level visual object classification (the object is a dog) and identification (it's my dog Bella). This is ongoing work with Pietro Perona at Caltech. The resulting algorithms are being applied to real-time machine vision.

We study the neuronal correlates of consciousness, developing a neurobiological framework to understand how subjective feelings (in particular, conscious visual perception) can arise in the mammalian forebrain. As we believe that top-down visual attention is a distinct neural process from the mechanisms underlying perceptual consciousness, we are experimenting with ways in which the effect of top-down attention can be dissociated from the effects of perceptually visibility.

231. Effects of a spatial and temporal non-uniform extracellular potential distribution on the activity of single neurons and neuronal populations

Costas Anastassiou[1], Christof Koch, Gyuri Buzs´aki[2], Mauricio Barahona[1]

While our knowledge of the dynamics and biophysics of synaptic and gap-junction communication has considerably increased over the last decades, the impact of non-synaptic electric field effects on cell signaling has been largely ignored despite experimental evidence that they exist. In fact, non-synaptic electric field

coupling between neurons has been related to a number of physiological and pathological states varying from integration in the mammalian olfactory system to motorneuron-activity in crushed nerves and epileptic seizures. In this project, we are concerned with the effects that the rapidly changing (>1 Hz) and spatially inhomogeneous LFP has on the transmembrane potential Vm of individual neurons. Much less is known about such rapid changes, partly because the local field potential (LFP) has usually not been characterized sufficiently well at a fine-grain spatial scale. Quantifying the impact of the LFP, both oscillatory and irregular, on Vm is crucial for understanding whether and how it influences neuronal activity. Despite the fact that this project is mainly computational, i.e., the biophysical modeling of model neuron representations will be conducted in the Koch laboratory at Caltech, experimental work will be carried out in the Buzs´aki laboratory at Rutgers who specializes in measurements of LFP in the hippocampus. Moreover, we shall be studying how such electric field coupling can affect the synchronization characteristics of neuronal assemblies. This last stage will be conducted in collaboration with Mauricio Barahona at Imperial College London, an expert in the analysis of networks and oscillatory behavior.

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232. A continuous attractor model for grid cell activity

Yoram Burak[*]*, Ila Fiete*

Measurements in the dorsolateral medial entorhinal cortex (dMEC) reveal that neurons selectively fire when a rat, moving around in an enclosure, visits any vertex of an imaginary regular triangular lattice that tiles the plane [1]. In this work we consider continuous attractor models as possible explanation for these findings.

Cortical connectivity, involving local excitation and surround inhibition, leads spontaneously to the formation of a triangular lattice pattern within a two-dimensional neural layer. Because all phases of the pattern are equivalent attractor states of the network, it's possible to induce rigid translations of the pattern using weak asymmetric weights within the network. Continuous attractor models of this type in the context of grid cells were suggested recently by us and other authors [2-4].

To test whether a continuous attractor model could reproduce grid cell dynamics, we simulated such models, subjecting the network to velocity inputs that mimic rat movements. We found that typically, in a finite network with non-periodic boundaries, a grid-cell firing pattern does not emerge (this observation is true for the recent model of Reference [2]. Also, see Reference [3]). To actually reproduce grid cell firing, stringent requirements must be met, which we enumerate. We show that if the network is finite with non-periodic boundaries, there are inherent reasons why it is difficult to meet these requirements.

We next show that a continuous attractor model may nevertheless yield grid cell firing patterns, in one of two possible ways. First, a proper choice of weights and external inputs can dramatically improve the network's response to velocity, allowing grid-cell firing to be reproduced. Nevertheless, the accumulation of errors with rat movement cannot be eliminated altogether even in the noise-free case. Second, in a network with periodic boundary conditions, the velocity response can be extremely accurate, limited in practice only by intrinsic noise or input errors. We suggest experimental tests to distinguish between these distinct possibilities - an important step in making concrete the idea of continuous attractors in neural computation.

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References

[1] T. Hafting *et al.* (2005) *Nature* **436**:801-806.
[2] M. Fuhs and D. Touretzky (2006) *J. Neurosci.* **26**:4266-4276.
[3] Y. Burak and I. Fiete (2006) *J. Neurosci*. **26**:9352-9354.
[4] B.L. McNaughton *et al*. (2006) *Nature Revs.* **7**:663-678.

233. Non-stereotyped odor responses in *Drosophila* mushroom body Kenyon cells

Mala Murthy[1]*, Ila Fiete, Gilles Laurent*[2]

The mushroom body is required for olfactory learning in flies. In adults, its principal neurons, the Kenyon cells (KCs), form a large cell population (~2,500 in *Drosophila*) usually divided into three classes α/β, α'/β', and γ), based on morphological and molecular criteria. Both the neurons from which they receive olfactory input, the antennal lobe projection neurons (PNs), and the presynaptic partners of the PNs, the olfactory sensory neurons, can be identified by their glomerular projections, the expression of specific genes, and their responses to odors. We ask here whether Kenyon cells are similarly identifiable. Focusing on a subset of ~23 genetically identified ab KCs, we recorded from neurons across flies, using whole-cell patch-clamp recordings *in vivo*. Odor responses across this subset provided no evidence for functionally stereotyped KCs across animals. Further, across-animal responses were as diverse within the genetically labeled subset as across all KCs in a larger data set. By contrast, replicate PNs showed stereotyped odor responses that could be clustered by glomerular identity. Using experimentally-derived PN responses as inputs to a simple KC model, we find that inter-fly PN response variability cannot alone explain the absence of KC response stereotypy. These results suggest differences in PN to KC connectivity across individual flies, with potential implications for how olfactory memory traces are formed within or read out from the MB.

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234. Observers are consistent when rating image conspicuity

Moran Cerf[1], Dan Cleary[1], Rob Peters[2], Wolfgang Einhäuser, Christof Koch

Human perception of an image's conspicuity depends on the stimulus itself and the observer's semantic interpretation. We investigated the relative contribution of the former, sensory-driven, component. Participants viewed sequences of images from five different classes - fractals, overhead satellite imagery, grayscale and colored natural scenes, and magazine covers - and graded each numerically according to its perceived conspicuity. We found significant consistency in this rating within and between observers for all image categories. In a subsequent recognition memory test, performance was significantly above chance for all categories, with the weakest memory for satellite imagery, and reaching near ceiling for magazine covers. When repeating the experiment after one year, ratings remained consistent within each observer and category, despite the absence of explicit scene memory. Our findings suggest that the rating of image conspicuity is driven by image-immanent, sensory factors common to all observers.

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235. Dynamic moment analysis of the extracellular electric field of a biologically realistic spiking neuron

Josh Milsten, Christof Koch

Based upon the membrane currents generated by an action potential in a biologically realistic model of a pyramidal, hippocampal cell within rat CA1, we perform a moment expansion of the extracellular field potential. We decompose the potential into both inverse and classical moments and show that this method is a rapid and efficient way to calculate the extracellular field both near and far from the cell body. The action potential gives rise to a large quadrupole moment that contributes to the extracellular field up to distances of almost 1 cm. This method will serve as a starting point in connecting the microscopic generation of electricfields at the level of neurons to macroscopic observables such as the local field potential.

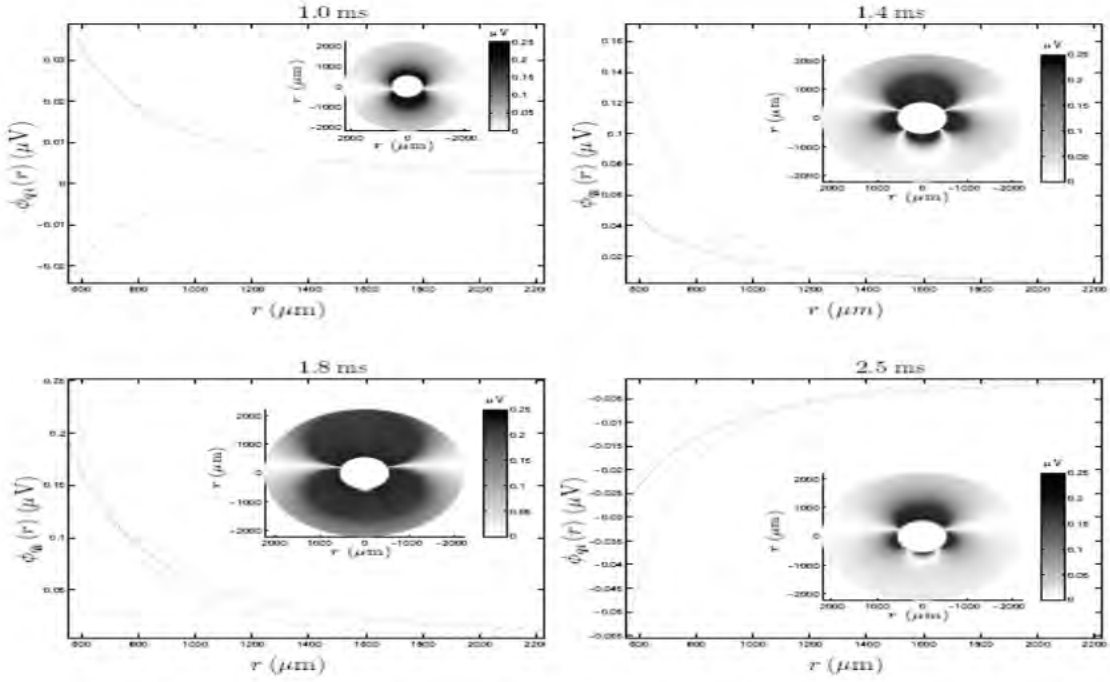

Figure 6: Moment comparison in the outer-field, about the peak in the action potential. The main figures show only the dominant dipole $l = 1$ (solid line) and quadrupole $l = 2$ (dashed line) contributions to the radial potential $\phi_q(r)$. The insets show the absolute value of the resulting total potential in μV. Excluded is the inner-field ($r < 550\mu$m) where the cell would be oriented along it's vertical axis as shown in Fig (3). Left to right, starting on the top, the times are given by 1.0, 1.4, 1.8 and 2.5ms.

236. Reward influences visual search behavior optimally, NIPS (submitted)

Vidhya Navalpakkam[*], Christof Koch, Pietro Perona[*]

Previous research shows that rare search targets like bombs in airport baggage screening are missed more often than frequent targets. Can we improve the detection rates in such critical searches? A simple model confirms that target frequency indeed will influence detection rates. However, the model predicts that the pattern of reward-punishment for correct detections and missed targets will also influence detection rates. We test this hypothesis on human subjects and find that indeed, reward schemes can be designed to improve detection performance significantly. Humans act as reward maximizing agents -- they deploy the optimal decision criterion that maximizes expected reward per trial. Thus, reward and target frequency influence human visual search behavior optimally.

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237. Dynamics of selective single neurons in the human MTL in a visual working memory task

Florian Mormann[1], Rodrigo Quian Quiroga[2], Alexander Kraskov[3], Moran Cerf[4], Itzhak Fried[1], Christof Koch

Single unit recordings in the human medial temporal lobe (MTL) have revealed neurons with selective, sparse, and invariant responses to different pictures of a given, familiar individual. We here used a working memory paradigm involving serial presentations of pictures at different presentation rates to investigate the dynamics of these neuronal responses.

We analyzed unit activity of MTL neurons from two patients undergoing monitoring for resective epilepsy surgery. Signals were recorded from microwires implanted in the MTL while patients performed a modified Sternberg task comprising a total of eight different pictures. Each trial consisted of a baseline period, an encoding period with presentation of a series of four images, a maintenance period, and a recall task in which the patient had to decide which one of two images he/she had seen in the previous series. Presentation rates of the picture series varied from 1-5 Hz using four different inter-stimulus intervals (ISIs). In total, 192 trials were randomized and balanced to yield sox presentations for each combination of image, ISI, and position within series. The evaluation of peri-stimulus time histograms of neurons responding selectively to only one of the images has yielded two types of responses. One group (10 neurons) showed early responses (with a latency of approximately 200 - 400 ms) independent of presentation rates. In another group (two neurons), responses occurred later (approximately 400 – 700 ms after stimulus onset) and were substantially diminished for fast presentation. Response dynamics did not appear to be influenced by the position of images within the presented series. For both groups there was no apparent correlation with task performance, but neurons in the second group tended to show selective activity during the maintenance period as well.

Results indicate distinct types of neurons with respect to their dynamical responses. The dynamics observed in the first group of neurons indicate an entirely stimulus-driven serial feed-forward processing for the investigated presentation rates, while those in the second group could reflect interference of consecutive processing for higher presentation rates, possibly caused by lateral inhibition. The activity of neurons during the maintenance period in the second group indicates an involvement of these MTL cells in working memory processing, which has previously been attributed primarily to prefrontal brain regions.

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238. Declarative memory formation in hippocampal sclerosis: An intracranial event-related potentials study

Florian Mormann[1,2], Guillen Fernandez[2], Peter Klaver[3,4], Bernd Weber[1,5], Christian E. Elger[1,5], Juergen Fell[1]

The functional deficits associated with hippocampal sclerosis during declarative memory formation are largely unknown. In this study, we analyzed intracranial event-related potentials recorded from the medial temporal lobes of nine epilepsy patients performing a word memorization task. We used frequency-specific wavelet analysis to assess stimulus-related changes in power and intertrial phase coherence. Statistical analysis revealed a significant decrease of stimulus-induced power in the δ and γ range on the side of pathology. No significant differences in phase locking were observed. Findings indicate a reduced availability of recruitable neural assemblies not only in the hippocampus but also in the rhinal cortex during memory formation. Network functions related to the timing of neural responses to the stimulus appear to be preserved.

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[5]*Department of NeuroCognition, Life & Brain Center, Bonn, Germany*

239. Measuring synchronization in coupled model systems: A comparison of different approaches

Thomas Kreuz[1,2,3], Florian Mormann[3], Ralph G. Andrzejak[2,4], Alexander Kraskov[2], Klaus Lehnertz[3,5], Peter Grassberger[2]

The investigation of synchronization phenomena on measured experimental data such as biological time series has recently become an increasing focus of interest. Different approaches for measuring synchronization have been proposed that rely on certain characteristic features of the dynamical system under investigation. For experimental data the underlying dynamics are usually not completely known, therefore, it is difficult to decide a priori which synchronization measure is most suitable for an analysis. In this study we use three different coupled model systems to create a 'controlled' setting for a comparison of six different measures of synchronization. All measures are compared to each other with respect to their ability to distinguish between different levels of coupling and their robustness against noise. Results show that the measure to be applied to a certain task cannot be chosen according to a fixed criterion but rather pragmatically as the measure that most reliably yields plausible information in test applications, although certain dynamical features of a system under investigation (e.g., power spectra, dimension) may render certain measures more suitable than others.

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240. Search goal tunes visual features optimally

Vidhya Navalpakkam, Laurent Itti[*]

How does a visual search goal modulate the activity of neurons encoding different visual features (e.g., color, direction of motion)? Previous research suggests that goal-driven attention enhances the gain of neurons representing the target's visual features. Here, we present mathematical and behavioral evidence that this strategy is suboptimal and that humans do not deploy it. We formally derive the optimal feature gain modulation theory, which combines information from both the target and distracting clutter to maximize the relative salience of the target. We qualitatively validate the theory against existing electrophysiological and psychophysical literature. A surprising prediction is that it is sometimes optimal to enhance nontarget features. We provide experimental evidence toward this through psychophysics experiments on human subjects thus, suggesting that humans deploy the optimal gain modulation strategy.

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Reference

Navalpakkam, V. and Itti, L. (2007) *Neuron* **53**(4):605-617.

241. Probabilistic modeling of eye movement data

Ueli Rutishauser[*], Christof Koch

Where the eyes fixate during search is not random; rather, gaze reflects the combination of information about the target and the visual input. It is not clear, however, what information about a target is used to bias the underlying neuronal responses. We here engage subjects in a variety of simple conjunction search tasks while tracking their eye movements. We derive a generative model that reproduces these eye movements and calculate the conditional probabilities that observers fixate, given the target, on or near an item in the display sharing a specific feature with the target. We use these probabilities to infer which features were biased by top-down attention: color seems to be the dominant stimulus dimension-guiding search, followed by object size and, lastly, orientation. We use the number of fixations it took to find the target as a measure of task difficulty. We find that only a model that biases multiple feature dimensions in a hierarchical manner can account for the data. Contrary to common assumptions, memory plays almost no role in search performance. Our model can be fit to average data of multiple subjects or to individual subjects. Small variations of a few key parameters account well for the inter-subject differences. The model is compatible with neurophysiological findings of V4 and FEP neurons and predicts the gain modulation of these cells.

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References

Rutishauser, U. and Koch, C. (2007) *J. Vision*, Volume 7, Issue 6, 1-20.

Einhäuser, W., Rutishauser, U., Frady, E.P., Nadler S., König, P. and Koch, C. (2006) *J. Vision*, Volume 6, Issue 11, 1148-1158.

242. Visual area MT+ responds to sound motion in early-blind human subjects

Melissa Saenz, Lindsay B. Lewis[1], Alex G. Huth, Ione Fine[2], Christof Koch

Over the last decade a series of brain imaging studies in humans have demonstrated that the visual cortex of people who become blind early in life responds to a variety of auditory, tactile, and verbal tasks. However, it remains unclear whether these cross-modal responses map onto the normal functional subdivisions of visual cortex found in sighted individuals. Here, using functional magnetic resonance imaging (fMRI), we tested whether visual cortical area MT+, well established for its role in visual motion processing in sighted subjects, is selectively recruited for auditory motion processing in early blind subjects.

We measured BOLD fMRI responses to moving vs. stationary auditory white noise stimuli in seven

normally sighted control subjects, five early-blind subjects, and two formerly (early) blind subjects with partial sight recovery (due to corneal stem cell replacement and cataract removal). Auditory motion stimuli were generated by varying inter-aural level differences (ILD) on stereo headphones. A sparse pulse sequence limited interference of MRI scanner noise with auditory stimuli. In the sighted control and sight recovery subjects we also measured responses to moving vs. stationary visual stimuli in order to functionally locate MT+. In early-blind subjects but not in sighted control subjects, we found that MT+ responded significantly to auditory motion. In sight recovery subjects MT+ responded to both auditory and visual motion.

These results show that in early-blind subjects visual area MT+ can maintain enhanced responses to motion, despite receiving input from a novel modality. This suggests an organizational principal behind cortical cross-modal plasticity, whereby reorganization is influenced by the normal functional role of a given region. In sight recovery subjects, we found that responses to auditory motion in visual area MT+ were at least as strong as the responses to visual motion. Thus, cross-modal responses can persist after sight recovery, coexisting with regained visual responses.

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243. Depth of interocular suppression associated with continuous flash suppression, flash suppression, and binocular rivalry

Naotsugu Tsuchiya[1], Christof Koch, Lee A. Gilroy[2] Randolph Blake[2]

When conflicting images are presented to the corresponding regions of the two eyes, only one image may be consciously perceived. In binocular rivalry (BR), two images alternate in phenomenal visibility; even a salient image is eventually suppressed by an image of low saliency. Recently, N. Tsuchiya and C. Koch (2005) reported a technique called continuous flash suppression (CFS), extending the suppression duration more than ten-fold. Here, we investigated the depth of this prolonged form of interocular suppression, as well as conventional BR and flash suppression (FS) using a probe detection task. Compared to monocular viewing condition, CFS elevated detection thresholds more than 20-fold, whereas BR did so by three-fold. In subsequent experiments, we dissected CFS into several components. By manipulating the number and timing of flashes with respect to the probe, we found that the stronger suppression in CFS is not due to summation between BR and FS but is caused by the summation of the suppression due to multiple flashes. Our results support the view that CFS is not a stronger version of BR but is due to the accumulated suppressive effects of multiple flashes.

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[2]*Department of Psychology, Vanderbilt University, Nashville, TN*

Reference

Tsuchiya, N., Koch, C., Gilroy, L. and Blake, R. (2006) *J. Vision* **6**(10):1068-1078.

244. Attention and consciousness: Two distinct brain processes

Christof Koch, Naotsugu Tsuchiya[*]

The close relationship between attention and consciousness has led many scholars to conflate these processes. This article summarizes psychophysical evidence, arguing that top-down attention and consciousness are distinct phenomena that need not occur together and that can be manipulated using distinct paradigms. Subjects can become conscious of an isolated object or the gist of a scene despite the near absence of top-down attention; conversely, subjects can attend to perceptually invisible objects. Furthermore, top-down attention and consciousness can have opposing effects. Such dissociations are easier to understand when the different functions of these two processes are considered. Untangling their tight relationship is necessary for the scientific elucidation of consciousness and its material substrate.

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245. Contrast thresholds for component motion with full and poor attention

Naotsugu Tsuchiya[1], Jochen Braun[2]

We compare luminance-contrast-masking thresholds for fully and poorly attended stimuli, controlling attention with a demanding concurrent task. We use dynamic displays composed of discrete spatiotemporal wavelets, comparing three conditions ("single," "parallel," and "random"). In contrast to static displays, we do not find that attention modulates the "dipper" regime for masks of low luminance contrast. Nor does attention alter direction-selective masking by multiple wavelets moving in random directions, a condition designed to isolate effects on component motion. However, direction-selective masking by multiple wavelets moving in parallel is significantly reduced by attention. As the latter condition is expected to excite both component and pattern motion mechanisms, this implies that attention may alter the visual representation of pattern motion. In addition, attention exhibits its well-known effect of reducing lateral masking between nearby spatiotemporal wavelets.

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Reference

Tsuchiya, N. and Braun, J. (2007) *J. Vision* **7**(3):1:1-15.

246. Sparse representation in the human medial temporal lobe

Stephen Waydo[1], Alexander Kraskov[2], Rodrigo Quian Quiroga[3], Itzhak Fried[4], Christof Koch

Recent experiments characterized individual neurons in the human medial temporal lobe with remarkably selective, invariant, and explicit responses to images of famous individuals or landmark buildings. Here, we used a probabilistic analysis to show that these data are consistent with a sparse code in which neurons respond in a selective manner to a small fraction of stimuli.

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[3]*University of Leicester, United Kingdom*
[4]*Univeristy of California, Los Angeles*

Reference

Waydo, S., Kraskov, A., Quian Quiroga, R., Itzhak, F. and Koch, C. (2006) *J. Neurosci.* **26**(40):10232-10234.

247. Explicit object representation by sparse neural codes

Stephen Waydo[*], Christof Koch

Highly sparse representations of objects in the visual environment in which individual neurons display a strong selectivity for only one or a few stimuli (such as familiar individuals or landmark buildings) out of on the order of 100 presented to a test subject have been observed in the human medial temporal lobe (MTL), a brain area believed to be crucial to the formation of new semantic memories. The process by which more distributed representations earlier in the visual pathway are transformed to produce such highly selective and invariant units results in information represented only implicitly by the pattern of light impinging on the retina and in the firing of neurons in early visual areas being made explicit at the level of MTL. This "sparsification" may be an important design principle underlying the structure of this brain region.

We apply a modified version of the model of Olshausen and Field, in which a network of nonlinear neurons generates a sparse representation of its inputs through an unsupervised learning process, to the outputs of a biologically plausible model of the human ventral visual pathway. We train this system on real-world images from multiple categories taken from the Caltech-256 dataset. This training is carried out in an entirely unsupervised manner, without specifying image categories or even the number of categories present. Although the underlying constraint in the model is merely to produce a sparse representation of its input set, units emerge that respond selectively to specific image categories such as faces and airplanes. The sparseness constraint thus, facilitates the formation of explicit representations of image categories, despite the category information being represented only implicitly in the input images.

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February (2007) poster, Computational and Systems Neuroscience (COSYNE).

248. The role of cortical interaction for tactile perception and its learning-induced changes

Claudia Wilimzig[*], Hubert R. Dinse[*]

Perceptual abilities are not fixed but subject to many factors such as practice, training, stimulation, and aging. We here provide a general framework to understand tactile spatial discrimination and its alterations during learning using a mean field approach. Cortical population activity is modeled with a Mexican hat interaction of short-range excitation and longer-range inhibition. Within such a representation tactile stimulation evokes either single peaks of activation coding for the subjective experience of a single point on the skin, while bimodal distributions are read out for two-points. While for large distances two peaks of activation interact only weakly, lateral interaction leads to substantial suppression for shorter ranges (Dinse and Jancke, 2002, *Prog. Brain Res.)*. For a critical metric distance between tactile stimuli this leads to monomodal activation profiles rather than bimodal ones, which is assumed to deteriorate the ability for two-point discrimination. Learning is assumed to lead to a decrease of inhibitory interaction. As a result, decreasing the strength of lateral inhibition in the model increases the size of the cortical representation as has been empirically observed in fMRI studies (Pleger *et al*., 2003, *Neuron*). The decrease in inhibitory interaction leads to bimodal activation profiles for this critical metric distance thus, explaining that learning improves two-point discrimination. The implications of decreased inhibition, which diminishes winner-takes-all mechanisms, can be seen in the emergence of flatter slopes in the psychometric functions.

There are other tasks such as localization, i.e., the ability to localize a stimulus in terms of absolute position on the skin. Interestingly, after training or coactivation there is a trade-off between two-point discrimination and localization abilities suggesting that competing mechanisms are involved in mediating these behaviors. We show that in the model decreased inhibition that improves two-point discrimination impairs localization performance showing dissociation between the role of inhibition for localization and discrimination. We conclude that inhibition plays a major role in determining the structure of population activations and that learning affects intra-cortical interaction.

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References

Dinse, H.R. and Jancke, D. (2002) *Prog. Brain Res.* **130**:155-173.

Pleger, B., Foerster, A.F., Ragert, P., Dinse, H.R., Schwenkreis, P., Nicolas, V. and Tegenthoff, M. (2003) *Neuron* **40**:643-653.

Publications

Carter, R.M., O'Doherty, J.P., Seymour, B., Koch, C. and Dolan, R.J. (2006) Contingency awareness in human aversive conditioning involves the middle frontal gyrus. *NeuroImage* **29**:1007-1012.

Cerf, M. Harel, J., Einhäuser, W. and Koch, C. Predicting human gaze using low-level saliency combined with face detection. *Neural Info. Process. Sys*. In press.

Cerf, M., Cleary, D.R., Peters, R.J., Einhäuser, W. and Koch, C. (2007) Observers are consistent when rating image conspicuity. *Vis. Res.* **47**:3052-3060.

Chiao, R.Y., Cohen, L.M., Leggett, A.J., Phillips, W.D. and Harper, Jr., C.L., eds., Cosmology and Consciousness. Cambridge University Press. In press.

Crick, F.C. and Koch, C.A. (2007) Neurobiological framework for consciousness. In: *The Blackwell Companion to Consciousness.* Velmans, M. and Schneider, S. eds., pp. 567-579, Blackwell, Oxford, UK.

Diba, K., Koch, C. and Segev, I. (2006) Spike propagation in dendrites with stochastic ion channels. *J. Comput. Neurosci*. **20**:77-84.

Dirk, W. and Koch, C. (2006) Modeling attention to salient proto-objects. *Neural Networks* **19**:1395-1407.

Einhäuser, W., Koch, C. and Makeig, S. (2007) The duration of the attentional blink in natural scenes depends on stimulus category. *Vision Res*. **47**:597-607.

Einhäuser, W., Kruse, W., Hoffmann, K.-P. and König, P. (2006) Differences of monkey and human overt attention under natural conditions. *Vision Res*. **46**:1194-1209.

Einhäuser, W., Mundhenk, T.N., Baldi, P., Koch, C. and Itti, L. (2007) A bottom-up model of spatial attention predicts human error patterns in rapid scene recognition. *J. Vision* **7**:1-13.

Einhäuser, W., Rutishauser, U., Frady E.P., Nadler S., König P. and Koch C. (2006) The relation of phase-noise and luminance-contrast to overt attention in complex visual stimuli. *J. Vision* **6**:1148-1158.

Frey, H.-P., König, P. and Einhäuser, W. (2007) The role of first- and second- order stimulus features for human overt attention. *Percept. & Psychophy*. **69**:153-161.

Gold, C., Henze, D.A., Koch, C. and Buzsáki, G. (2006) On the origin of the extracellular action potential waveform. *J. Neurophysiol.* **95**:3113-3128.

Harel, J. Koch, C. and Perona, P. Graph-based visual saliency. Neural Information Processing Systems, NIPS, 2006 conference paper.

Kanai, R., Tsuchiya, N. and Verstraten, F.A.J. The scope and limits of top-down attention in unconscious visual. *Curr. Biol*. In press.

Koch, C. and Greenfield, S. (2007) How does consciousness happen? *Sci. American*, **297**(4):76-83.

Koch, C. and Hepp, K. (2006) Quantum mechanics in the brain. *Nature* **440**:611-612.

Koch, C. and Hepp, K. (2006) The relation between quantum mechanics and higher brain functions: Lessons from quantum computation and neurobiology. In: *Amazing Light: Visions for Discovery: New Light on Physics*. *Nature* **440**:611-612.

Koch, C. and Preuschoff, K. (2007) Betting the house on consciousness. *Nature Neurosci.* **10**:140-141.

Koch, C. and Tononi, G. (2007) Consciousness. In: *New Encyclopedia of Neuroscience,* Elsevier. In press.

Koch, C. and Tsuchiya, N. (2007) Attention and consciousness: Two distinct brain processes. *Trends Cogn. Sci.* **11**:16-22.

Koch, C. The neuroscience of consciousness. In: *Fundamental Neuroscience*, 3rd edition. Squire, L., editor, Elsevier. In press.

Kraskov, A., Quian Quiroga, R., Reddy, L., Fried, I. and Koch, C. (2006) Local field potentials and spikes in the human medial temporal lobe are selective to image category. *J. Cogn. Neurosci.* **19**:479-492.

Kreiman, G., Hung, C.P., Kraskov, A., Quian Quiroga, R., Poggio, T. and DiCarlo, J. (2006) Object selectivity of local field potentials and spikes in the macaque inferior temporal cortex. *Neuron* **49**:433-445.

Milstein, J. and Koch, C. Dynamic moment analysis of the extracellular electric field of a biologically realistic spiking neuron. *Neural Comput*. In press.

Mlodinow, L. (2006) Chaotic: How Hollywood really works. *Los Angeles Times Sunday Magazine*.

Moradi, F., Hipp, C. and Koch, C. (2007) Activity in the visual cortex is modulated by top-down attention locked to reaction time. *J. Cogn. Neurosci.* **19**:331-340.

Navalpakkam, V. and Itti, L. (2007) Search goal tunes visual features optimally. *Neuron* **53**(4):605-617.

Quian Quiroga, R., Reddy, L., Koch, C. and Fried, I. Decoding visual inputs from multiple neurons in the human temporal lobe. *J. Neurophysiol.* In press.

Reddy, L., Moradi, F. and Koch, C. Top-down biases win against focal attention in the fusiform face area. *Neuroimage*. In press.

Reddy, L., Quian Quiroga, R., Wilken, P., Koch, C. and Fried. A. (2006) Single neuron correlate of change detection and change blindness in the human medial temporal lobe. *Curr. Biol.* **16**:2066-2072.

Reddy, L., Reddy, L. and Koch, C. (2006) Face identification in the near-absence of focal attention. *Vis. Res*. **46**:2336-2343.

Rutishauser, U. and Koch, C. (2007) Probabilistic modeling of eye movement data during conjunction search via feature-based attention. *J. Vision* **7**:1-20.

Tsuchiya, N. and Braun, J. Contrast thresholds for component motion with full and poor attention. *J. Vision*. In press.

Tsuchiya, N., Koch, C., Gilroy, L.A. and Blake, R. (2006) Depth of interocular suppression associated with continuous flash suppression, flash suppression, and binocular rivalry. *J. Vision* **6**:1068-1078.

VanRullen, R., Reddy, L. and Koch, C. (2006) The continuous wagon wheel illusion is associated with changes in electroencephalogram power at ~13Hz. *J. Neurosci.* **26**:502-507.

Waydo, S. and Koch, C. Unsupervised learning of individuals and categories from images. *Neural Comput.* In press.

Waydo, S., Kraskov, A., Quian Quiroga, R., Fried, I. and Koch, C. (2006) Sparse representation in the human medial temporal lobe. *J. Neurosci.* **26**:10232-10234.

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Summary: The direction of our neurophysiological studies has been influenced by the inclusion and collaboration of postdoctoral fellows whose background is in hard sciences. Barn owls can pinpoint a rustling mouse in total darkness. Their ears are designed to localize sound in both the horizontal and vertical directions. Their brain contains neural circuits that can compute time differences between the ears as small as 10 μ sec. Ashida *et al.* (2007) modeled neurons responsible for the detection of such small time differences.

249. Passive soma facilitates submillisecond coincidence detection in the owl's auditory system

Ashida, G., Abe, K., Funabiki, K., Konishi, M.

Neurons of the avian nucleus laminaris (NL) compute the interaural time difference (ITD) by detecting coincident arrivals of binaural signals with submillisecond accuracy. The cellular mechanisms for this temporal precision have long been studied theoretically and experimentally. The myelinated axon initial segment in the owl's NL neuron and small somatic spikes observed in auditory coincidence detector neurons of various animals suggest that spikes in the NL neuron are generated at the first node of Ranvier and that the soma passively receives back-propagating spikes. To investigate the significance of the "passive soma" structure, we constructed a two-compartment NL neuron model, consisting of a cell body and a first node, and systematically changed the excitability of each compartment. Here, we show that a neuron with a less active soma achieves higher ITD sensitivity and higher noise tolerance with lower energy costs. We also investigate the biophysical mechanism of the computational advantage of the "passive soma" structure by performing sub- and suprathreshold analyses. Setting a spike initiation site with high sodium conductance, not in the large soma but in the small node, serves to amplify high-frequency input signals and to reduce the impact and the energy cost of spike generation. Our results indicate that the owl's NL neuron uses a "passive soma" design for computational and metabolic reasons.

We previously had shown that neurons in the external nucleus of the inferior colliclus in the midbrain multiply neural codes for time and intensity. These neurons do not respond to a time or an intensity difference alone but require their combination, which can be obtained by multiplying them. Fischer *et al.* (2007) found that neurons in the last station before the external nucleus multiply incoming synaptic signals, although some neurons were not perfect multipliers.

250. Emergence of multiplicative auditory responses in the midbrain of the barn owl

Fischer, B.J., Peña, J.L. Konishi, M.

Space-specific neurons in the barn owl's auditory space map gain spatial selectivity through tuning to combinations of the interaural time difference (ITD) and interaural level difference (ILD). The combination of ITD and ILD in the subthreshold responses of space-specific neurons in the external nucleus of the inferior colliculus (ICx) is well described by a multiplication of ITD- and ILD-dependent components. It is unknown, however, how ITD and ILD are combined at the site of ITD and ILD convergence in the lateral shell of the central nucleus of the inferior colliculus (ICcl) and therefore whether ICx is the first site in the auditory pathway where multiplicative tuning to ITD- and ILD-dependent signals occurs. We used extracellular recording of single neurons to determine how ITD and ILD are combined in ICcl of the anesthetized barn owl. A comparison of additive, multiplicative, and linear-threshold models of neural responses shows that ITD and ILD are combined nonlinearly in ICcl, but the interaction of ITD and ILD is not uniformly multiplicative over the sample. A subset (61%) of the neural responses is well described by the multiplicative model, indicating that ICcl is the first site where multiplicative tuning to ITD - and ILD-dependent signals occurs. ICx, however, is the first site where multiplicative tuning is observed consistently. A network model shows that a linear combination of ICcl responses to ITD–ILD pairs is sufficient to produce the multiplicative subthreshold responses to ITD and ILD seen in ICx.

We continue our work with zebra finches. We pursued our work on one of the song control areas called Uva. Despite its small size and location, it turned out to be a very interesting area. Neurons in all song control areas are supposed to receive auditory input from forebrain auditory center Field L and it projection areas. This conjecture is largely based on anatomical studies. We found that lesions of Uva eliminate auditory responses in a song control area called HVC from which all other song areas receive direct and indirect inputs. We are currently trying to show that Uva is the only site by which auditory input enters the song control system.

Publications

Ashida, G., Abe, K., Funabiki, K. and Konishi, M. (2007) Passive soma facilitates submillisecond coincidence detection in the owl's auditory system. *J. Neurophys.* **97**(3):2267-2282.

Fischer, B.J., Peña, J.L. and Konishi, M. (2007) Emergence of multiplicative auditory responses in the midbrain of the barn owl**.** *J. Neurophysiol.* **98**:1181-1193.

Lawrence A. Hanson Jr. Professor of Biology and Computation and Neural Systems: Gilles Laurent
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Summary: We are interested in information coding in the brain and in the design principles of circuits involved in processing sensory information. We are particularly interested in understanding the role of time, circuit dynamics, synchronization and oscillations in information coding and in relating the biophysical properties of neurons and synapses to the function of the networks in which they are embedded. We therefore study the cellular, synaptic and network aspects of neural processing. We continued to focus our research this year on the olfactory system of insects (antennal lobes and mushroom bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices), using locusts, Drosophila and crickets as primary model systems, and of zebrafish (olfactory bulb). Our work combines experimental (behavioral, electro-physiological and two-photon imaging) and modeling techniques and aims at understanding functional aspects of brain circuit design, such as the coding and learning rules used by the nervous system.

251. Criticality, short term-memory, and optimization of computation in an olfactory system

Ingmar Riedel-Kruse

I wish to understand how neuronal networks can learn to perform computational tasks optimally by exploiting properties of their network design and neuronal dynamics. This is a central question in systems neurobiology.

I study the antennal lobe (AL) in the locust olfactory system - a neuronal network carrying out highly dynamic odor discrimination tasks. Repeated odor encounters tune the neurons in the AL towards an odor-specific synchronous oscillatory firing pattern. This tuning is a form of unsupervised learning (or short-term memory). Transient spontaneous oscillations are still present when the odor stimulus is absent, which strongly suggests that this system is close to a criticality.

Systems close to criticality show substantial non-linear behavior and have therefore been proposed to be optimal sensors with high sensitivity and large dynamic range. One example for this proposition is hearing, where the self-tuning mechanism of the hair-bundle is close to an oscillatory instability.

I test experimentally whether the AL truly utilizes criticality, investigate how criticality facilitates odor discrimination and memory formation, and develop a theoretical model to understand advantages and limitations of criticality in the AL. I use single and multi-neuronal recording techniques, and combine them with theoretical approaches of non-linear dynamic systems.
My work aims for conceptual new insight into olfactory processing, memory formation and the role of the widely observed oscillatory activity in neuronal networks.

References

Bak, P., Tang, C. and Wiesenfeld, K. (1988) *Phys. Rev.* **A38**:364-374.
Camalet, S., Duke, T., Julicher, F. and Prost, J. (2000) *Proc. Natl. Acad. Sci. USA* **97**:3183-3188.
Mazor, O. and Laurent, G. (2005) *Neuron* **48**:661-673.
Pikovsky, A., Rosenblum, M. and Kurths, J. (2003) *SYNCHRONIZATION – A Universal Concept in Nonlinear Sciences.* Series: Cambridge Nonlinear Science Series, (No 12), Cambridge University Press, Cambridge, UK.
Stopfer, M. and Laurent, G. (1999) *Nature* **402**:664-668.
Strogatz, S.H. (1994) *Nonlinear Dynamic and Chaos: With Applications to Physics, Biology, Chemistry, and Engineering.* In: Perseus Books, Westview Press, Cambridge, MA.
Wilson, R.I., Turner, G.C. and Laurent, G. (2004) *Science* **303**:366-370.

252. Wide sampling of odor-representation space in the locust antennal lobe

Benjamin D. Rubin

With its relatively small and well-characterized olfactory system, the locust (*Schistocerca americana*) provides an experimentally advantageous system for understanding how the brain represents, transforms and stores information about sensory stimuli. Olfactory receptor neurons in the antenna project to the antennal lobe, where excitatory projection neurons (PNs) and inhibitory non-spiking local neurons interact to yield complex odor response dynamics. These interactions give rise to a 20-30 Hz local field potential (LFP) that reflects the transient synchronization of ensembles of excited PNs whose membership evolves with each oscillation cycle. While the existence of these diverse cell- and odor-specific response patterns is well established, less is known about the extent to which the responses of individual and

populations of PNs are constrained. Such constraints exist because, as members of a densely interconnected neuronal network, the activity of any one PN depends in part on the responses of other PNs. To address this issue, we are studying the activity of a large fraction of the ~800 PNs that populate each AL in response to a large set of odors (~50). Using silicon probes to acquire extracellular recordings of PNs and simultaneously monitor the LFP *in vivo*, we have so far recorded from well over 100 PNs in response to a panel of ~50 monomolecular odors that vary in their structural similarity. Most PNs respond to both the mechanical and chemical components of a stimulus, with distinct response patterns for different odors. On average, trajectories representing the PN population responses to different odors diverge and then converge back to their baseline state with roughly the same time course as the mean firing rate changes across the population. The functional topography of coding space in the AL is being explored.

253. Plasticity and decoding of sparse representations in the locust mushroom body

Stijn Cassenaer, Gilles Laurent

The insect mushroom body (MB) is used as a model system to address several questions of general neurobiological interest such as sensory discrimination, multi-modal integration, the control of complex behavioral repertoires, as well as learning and memory (1). Among these, the role of the MB in olfaction and memory has received considerable attention. A number of studies have addressed how olfactory information reaches the MB and how it is transformed along the way (2,3). A particularly dramatic transformation occurs between the antennal lobe (AL) and the MB. Whereas odor representations in the AL are densely distributed and dynamic, only one synapse further downstream, odor representations are sparse, comprised of brief activation of a small fraction of the MB intrinsic neurons (Kenyon cells, KCs). This sparse representation should be advantageous for learning and memory, as it reduces the number of synapses that need to be modified and the number of comparisons necessary for pattern matching (4). There are several lines of evidence that implicate the synaptic contacts between KCs and their downstream targets in memory storage and recall (5).

In order to address the question of how the sparse representations carried by KCs are decoded and further transformed before leaving the MB, we are investigating the cells that take KC activity as their input, the MB extrinsic cells. There appear to be several distinct cell types within this population that differ most obviously with respect to the neuropils they invade (6,7). We are characterizing these neurons in terms of their physiological and morphological properties by means of intracellular and tetrode recordings, as well as extracellular stimulation and pressure-microinjection. We are investigating how odors are represented by this population, how efficacious the KC-extrinsic cell synapses are, how they can be modified, and how the extrinsic cells integrate their KC inputs.

We have found that these synapses are subject to Hebbian, spike-timing dependent plasticity (STDP), which contributes to the synchronous flow of olfactory information through the MB (8).

References

1. Strausfeld, N., Hansen, L., Yongsheng, L., Gomez, R.S. and Ito, K. (1998) *Learning & Memory* **5**:11-37.
2. Wilson, R.I., Turner, G.T. and Laurent, G. (2004) *Science* **303**:366-370.
3. Perez-Orive, J., Mazor, O., Turner, G.T., Cassenaer, S., Wilson, R.L. and Laurent, G. (2002) *Scienc*e **297**:359-365.
4. Laurent, G. (2002) *Nature Revs. Neurosci.* **3**:884-895.
5. Heisenberg, M. (2003) *Nature Revs. Neurosci*. **4**:266-275.
6. MacLeod, K. Backer, A. and Laurent, G. (1998) *Nature* **395**:693-698.
7. Farivar, S. (2005) Ph.D. thesis, Caltech.
8. Cassenaer, S. and Laurent, G. (2007) Nature advance online publication (doi:10.1038/nature05973).

254. Non-stereotyped odor responses in *Drosophila* mushroom body Kenyon cells

Mala Murthy

Over the past year, I have investigated the logic of neuronal connectivity in the *Drosophila* mushroom body, a higher order brain center required for complex processes such as learning and memory. The mushroom body (MB) is important for olfactory learning: molecules implicated in learning are concentrated in the MB, mutants that disrupt these molecules have defects in memory formation, and anatomical studies have shown that experience can induce substantial structural transformations. Such plasticity suggests that odor representations by Kenyon cells (KCs), the neurons that comprise the MB, by their targets, or both, could change within the lifetime of each animal or across animals. Further, KCs belong to large populations (2,500 in *Drosophila*; 300,000 in cockroaches; millions in the related arthropod *Limulus*) of similar neurons: morphological and molecular subtypes have been described, but identifiable individuals have not. Yet, the neurons from which they receive olfactory input, the projection neurons (PNs), are unambiguously identifiable, by virtue of their restricted projections to individual antennal lobe glomeruli, expression of molecular markers, and odor responses. In the *Drosophila* MB, there is conflicting evidence regarding anatomical and functional stereotypy, and it is not known if the matrix of connections between PNs and KCs is predetermined, thus giving rise to stereotyped odor responses within identifiable KCs, or if this matrix of connections is more random, thus varying across individuals. The extent to which KCs are deterministically stereotyped affects how experience and learning may modify odor representations within the MB. To address this question of stereotypy, I conducted *in vivo* whole-cell patch-clamp recordings from a subpopulation of labeled KCs in *Drosophila melanogaster*, in order to

record from the same cell or cells across genetically identical flies.

In the fruit fly, the mushroom body is derived from four neuroblasts, and by divisions through the embryonic, larval and pupal stages, each neuroblast generates ~500 neurons per clonal unit. Each cluster can be subdivided into three KC classes (α/β, γ, and α'/β') based on adult axonal projection patterns and birth order. The *Drosophila* GAL4/UAS system allows for spatial control over transgene expression, based on the promoter regulating the GAL4 insert. GAL4 line *NP7175* is expressed in a small subset of KCs (23.6±3.3 KCs per neuroblast clonal unit; 20 clonal units counted) that project to the core of the α/β lobes. Moreover, each of the four clonal units can be distinguished based on the position of somata and the region of the MB calyx innervated by their dendrites, which allows for identification of the same ~23 cells across flies. In order to sample at least six replicate KCs, I have recorded from 22 KCs from the left hemisphere, lateral posterior (L-LP) clonal unit of *NP7175-GAL4; UAS-eGFP2x/Cyo* one day-old females, raised under identical conditions.

Odor responses across this subset provided no evidence for functionally stereotyped KCs across animals. Further, across-animal responses were as diverse within the genetically labeled subset as across all KCs in a larger data set. By contrast, recordings from replicate PNs showed stereotyped odor responses that could be clustered PN-to-KC convergence and experimentally derived PN responses as inputs to simple KC models, we find that inter-fly PN response variability cannot alone explain the absence of KC response stereotypy. These results suggest differences in PN to KC connectivity across individual flies, with potential implications for how olfactory memory traces are formed within or read out from the MB.

The olfactory system of *Drosophila* is becoming one of the best-characterized sensory systems in large. Molecular, anatomical and physiological analyses indicate that its antennal lobe circuits are so precisely organized that olfactory sensory neurons and projection neurons can both be identified using any of the above characteristics (alone or in combinations). Using electrophysiological recordings and cell-specific genetic markers, we assessed whether such precise circuit specification continues in the mushroom bodies, an important issue because: (i) mushroom bodies are required for olfactory learning and recall, and thus responses within this structure might vary across individuals; and (ii) because KCs are very numerous (~2,500 in *Drosophila*, several hundred thousands in large insects, millions in larger arthropods), making the precise specification of pairwise connectivity a significant mechanistic challenge. We failed to find identifiable response profiles among a small subset of 23 genetically labeled KCs, suggesting that one-to-one connections between PNs and KCs vary from animal to animal.

255. Reliability and conditional probabilities of projection neuron responses

John M. Delacruz

In recent years, work from the Laurent lab has revealed much about the temporal dynamics and response properties of antennal lobe projection neurons (PNs) and their downstream targets, mushroom body Kenyon cells (KCs). However, many details remain to be understood regarding how information is transformed from the PNs to the KCs. Two questions which may help us in better understanding this transformation are: 1) How reliable are the PNs in terms of the information they carry? 2) What is the functional connectivity between PNs? We are addressing both of these questions using the same experimental paradigm. One-second odor pulses are delivered to the antennae of a restrained locust while simultaneously recording from a population of PNs using an extracellular tetrode. The same odor is presented once per 20 sec trial for several hundred trials. By replicating the stimulus paradigm several hundred times, we are able to assess the reliability of the PN response in greater detail than has been attempted in the past. The functional connectivity of PNs will be assessed by looking at pairwise response correlations and by analyzing the conditional probability of PN responses (given that PN 1 fires an action potential at a given time point, what is the probability that PN 2 fires an action potential at the same or some later time point?). We expect that in addressing both these questions we will be on step closer to understanding how olfactory information is processed in the insect brain.

256. Feedback inhibition in the mushroom body and gain control

Maria Papadopoulou, Glenn Turner[*]*, Gilles Laurent*

The giant GABAergic neuron (GGN) is a single, paired, non-spiking neuron that arborizes extensively in the mushroom body (MB) (Leitch and Laurent, 1996), where it overlaps with the dendrites and the axons of Kenyon cells (KCs). KCs are the intrinsic neurons of the MB and are thought to be required for learning and memory (Heisenberg, 2000). We are interested in understanding the function of GGN in olfactory processing: in particular, its pattern of arborization makes it an attractive candidate for controlling or modulating KC responses to odors, with potential implications for learning and recall. Physiological recordings of KCs in locust show that these neurons respond sparsely to odors, in contrast to their excitatory input from antennal lobe projection neurons (PNs) (Perez-Orive *et al.*, 2002). Inhibition appears to be critical to control KC response threshold, probability and duration during odor stimulation (Perez-Orive *et al*., 2002). While a source of feedforward inhibition has been identified, we suspect that GGN, because of its GABAergic output, contributes to feedback inhibitory control of KC excitability. As such, this neuron could act to control the gain of PN-to-KC information transfer and normalize KC-population output, making it

independent of input strength. Using electrophysiological techniques, we are studying the properties and modes of action of GGN in locust, and in its functional equivalent in *Drosophila*.

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References

Leitch, B. and Laurent, G. (1996) *J Comp Neurol.* **4**:487-514.

Heisenberg, M. (2000) *Nat Revs. Neurosci.* **4**:266-275.

Perez-Orive, J., Mazor, O., Turner, G.C., Cassenaer, S., Wilson, R. and Laurent, G. (2002) *Science* **297**:359-365.

257. Plasticity of olfactory representations in zebrafish larvae

Jonathan Young

We are interested in the question of olfactory circuit plasticity: does the representation of an odor object change over the life history of the animal due to experience and development, and what impact does this have on olfactory computation? The zebrafish larva provides a vertebrate model system for studying olfaction that offers the advantages of complete transparency, complex behavior, established genetics, and external development, which are the properties needed to investigate this question. Work has previously been done in this lab to characterize the responses of mitral cells in the adult zebrafish olfactory bulb. We will use the larval form of the zebrafish to study olfactory learning and circuit development.

Using a combination of behavioral, electrophysiological, and calcium imaging techniques, we will investigate the capabilities of the larval olfactory system to learn and change through development, and observe how these changes are encoded in both the activity of the olfactory bulb and the behavioral responses of the animal.

We have developed an aversive olfactory conditioning paradigm in the zebrafish larvae, which enables us to induce learning in the brain while simultaneously recording neuronal activity using two-photon calcium imaging. This method will allow us to observe any changes induced by learning that occur in the activity of the mitral cells encoding odor representations. We will test the hypothesis that the olfactory bulb acts as a tunable filter, improving its performance in speed and accuracy to conditioned odors that are of relevance to the animal.

258. Transformation of olfactory representations across morphed odor conditions in the antennal lobe and the mushroom body

Kai Shen, Gilles Laurent

In nature, animals rarely encounter stimuli in isolation and must often extract meaningful information from complex odor mixtures. And unlike vision, where we can easily parse out the individual lines in a Kandinsky painting, in olfaction, we typically perceive odors as single entities. Behaviorally, it has been shown that human subjects can rarely identify the individual components within a mixture when there are four or more components present [1]. On the other extreme, the female malaria mosquito can detect a single chemical 4-methylphenol, which is present in our sweat. How then, does the brain achieve these two seemingly contradictory tasks? We begin to address these questions using electrophysiological approaches, by recording from principal neurons of the antennal lobe (PNs) and the mushroom body, the first and second relays of the locust olfactory system.

Individual PNs respond with characteristic epochs of excitation and inhibition that are both neuron- and odor-specific [2]. And because not all responding neurons express the same patterns at the same time, the state of the network is dynamic, carried by an assembly of neurons that evolves in a stimulus-specific manner over time. Neural representations can thus be described geometrically as stimulus-specific trajectories reflecting the state of the PN network [3]. How do these trajectories change with small changes in the stimulus? Do the trajectories change progressively to reflect changes in the stimulus, or are there abrupt discontinuous transitions from one trajectory to another? We addressed this question by first probing the PN network with small changes to the input that varied progressively along a continuum. By varying the ratio of concentrations of two components within a binary mixture, we effectively morphed one unique odor to another. We observed a progressive rather than abrupt transformation from one odor-specific trajectory to another.

To observe the transformation across a more broad range of stimulus space, we systematically increased the complexity of the mixture by adding single odorants in a stepwise manner from two to eight (e.g., AB, ABC, ABCD, → ABCDWXYZ) and presenting different combinations that overlap by varying amounts (e.g., ABCD, ABCX, BCWX, BDWX, DWYZ, WXYZ). To test the linearity of mixture representation, we estimated the ensemble PN response vectors to odor mixtures by adding the response vectors corresponding to the mixture components. We then examined the degree of deviation between these estimated response vectors and the experimentally observed response vectors. We find that for binary mixtures, there is good approximation between the estimated and experimental response vectors, but this approximation degrades rapidly when more odorants are added to the mixture. Finally, we examine the degree to which these representations mirror the responses of the targets of the PNs in the mushroom body, the Kenyon cells.

References

[1] Laing, D.G. and Francis, G.W. (1989) *Physiol. & Behav.* **46**:809-814.

[2] Laurent, G. Wehr, M. and Davidowitz, H. (1996) *J. Neurosci.* **16**:3837-3847.

[3] Stopfer, M. Jayaraman, V. and Laurent, G. (2003) *Neuron* **39**:991-1004.

259. Transfer at *C. elegans* synapses

Anusha Narayan

A variety of techniques have been used to dissect neural circuits in *C. elegans*. These include light and electron microscopy, focal laser ablations, and calcium imaging. For a better functional understanding of these circuits, however, we need to know the rules of synaptic information transfer. How is the dynamic range of the post-synaptic neuron set? What are the mechanisms for synaptic integration and gain control? Questions such as these can best be answered by monitoring or controlling connected pre- and post-synaptic neurons simultaneously.

We focus on the synapse between the AFD/ASER and AIY neurons, since the functional relevance of these neurons has been established and there is anatomical evidence for synapses between them. Channelrhodopsin-2 (chR2) is a light activated cation channel with fast kinetics (order of milliseconds[1]). We express chR2 under a neuron-specific promoter[2] in the presynaptic neuron, and use whole-cell patch-clamp recording techniques to monitor membrane voltage or currents in the postsynaptic neuron. We are first calibrating the response to light of chR2-expressing neurons. Currently, we are calibrating this light response in worms expressing chR2 in ASER. We have observed depolarizations of 10-30 mV in response to light (450-490 nm) in current clamp, and inward currents of 5-10 pA in voltage-clamp. We have also seen evidence of spontaneous synaptic activity, in the form of discrete synaptic events (potentials or currents) with different reversal potentials. We are beginning to characterize the ASER-AIY synapse, and will then move on to the AFD-AIY synapse.

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References

[1]Boyden, E.S., Zhang, F., Bamberg, E., Nagel, G. and Deisseroth, K. (2005) *Nature Neurosci.* **8**:1263-1268.

[2]Nagel, G., Brauner, M., Liewald, J.F., Adeishvili, N., Bamberg, E. and Gottschalk, A. (2005) *Curr. Biol.* **15**:2279-2284.

260. Functional organization of the central complex in an insect brain

Cindy Chiu[*]

The central complex is a midline neuropil brain structure that is present in most insects. Its most striking feature is a three-dimensional matrix structure which interconnects the left and right hemispheres through an ordered set of chiasmata[1,2]. Although the intrinsic anatomy of the central complex is well mapped, the input-output connectivity of this region is poorly understood. Based on behavioral studies of *Drosophila melanogaster* central complex mutants and a small number of electrophysiology recordings in locusts, the central complex has been implicated in "higher order" brain functions including integration of visual sensory information and regulation of motor-related activities[3,4,5]. Additionally, its systematic inter-hemispheric connectivity points to a role in mapping external space and integrating this information across the left and right hemispheres. Despite these tantalizing possibilities, the essential function of the central complex remains elusive.

My goal is to elucidate the functional organization of the central complex. To date, there are few electrophysiological data of central complex neurons. Given the broad range of possible inputs to this structure, I have chosen a battery of diverse, ethologically relevant stimuli. Using this broad-based stimulus repertoire, I aim to: (1) characterize using intracellular methods the electrophysiological and anatomical properties of individual central complex neurons; (2) examine the physiological responses of central complex neurons during orientation behaviors; and, (3) investigate the circuit properties of the central complex system by recording from neural ensembles. I have chosen for my model organism the field cricket, *Gryllus bimaculatus*, because it is amenable to *in vivo* intracellular and extracellular electrophysiology methods, and it exhibits robust behaviors that can be studied in laboratory conditions[6]. Recordings thus far have revealed multimodal responses in putative central complex neurons.

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References

[1]Williams, J.L.D. (1975) *J. Zool. Lond*. **176**:67-86.

[2]Hanesch, U., Fischbach, K.F. and Heisenberg, M. (1989) *Cell Tissue Res.* **257**:343-366.

[3]Heisenberg, M. (1994) In: *Neural Basis of Behavioral Adaptations*. *Adv. Zool*. **39**:61-79.

[4]Strauss, R. (2002) *Curr. Op. Neurobiol.* **12**:633-638.

[5]Homberg, U. (2004) *Naturwissenschaften* **91**:199-208.

[6]Huber, F., Moore, T.E., Loher, W., Eds. (1989) *Cricket Behav. & Neurobiol.*

261. Olfactory processing during locust antennal movements

Stephen J. Huston

I am investigating how the processing of sensory information changes when an animal actively investigates its environment. Previous work in the Laurent lab has been successful in using neural signals recorded from the locust olfactory system to provide insights into how brains encode sensory information. However, these experiments were performed on locusts with restrained antennae, when we know from observation that during normal function locusts move their antennae to actively sample odors.

I am beginning a new set of experiments to determine how antennal movements affect olfactory processing. I have developed equipment to record the direction of walking and antennal movements simultaneously from a tethered locust. I am in the process of characterizing the locust's antennal responses to odor presentation (**Figure 1**). Once the behavioral characterization is completed, my aim is to record neural activity from the locust olfactory system while the locust is free to move its antennae. Can we predict the neural responses to olfactory stimuli from previous experiments

on restrained locusts, or does neural processing in the olfactory system change as the locust moves its antennae to investigate the environment?

This work will move beyond the study of sensory systems as passive entities, allowing us to study neural activity while the animal actively samples the sensory world.

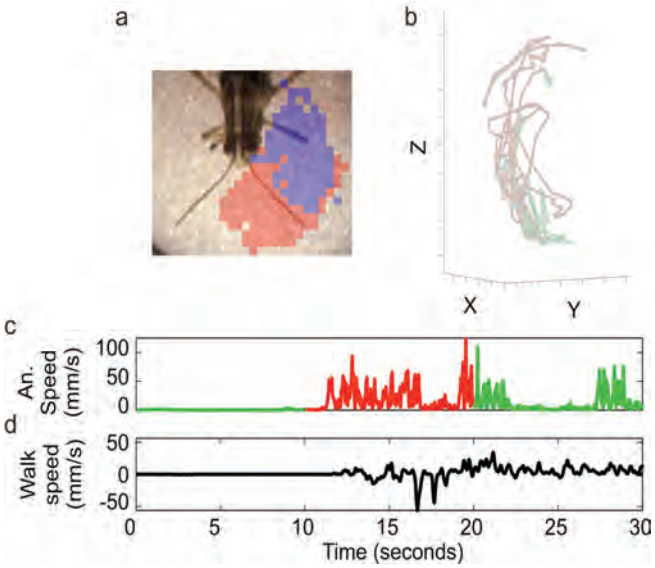

Figure 1. (**a**) Top view of the antennae of a tethered locust. Superimposed red and blue squares indicate whether the locust's left antenna is more likely to be at the given location when a grassy odor is presented from the locust's left (blue) or right (red). (**b**) 3D position of the left antennal tip before (green), during (red) and after (green) odor presentation. (c) Speed of the antenna plotted in (**b**), red indicates the duration of odor presentation. (**d**) Walking speed of the locust during the trial.

Publications

Assisi, C., Stopfer, M., Laurent, G. and Bazhenov, M. (2007) Adaptive regulation of sparseness by feedforward inhibition. *Nat Neurosci* **10**: 1176 - 1184.

Broome, B.M., Jayaraman, V. and Laurent, G. (2006) Encoding and decoding of overlapping odor sequences. *Neuron* **51**:467-482.

Cassenaer, S. and Laurent, G. (2007) Hebbian STDP in mushroom bodies facilitates the synchronous flow of olfactory information. *Nature* **448** (7154): 709-713.

Jayaraman, V. and Laurent, G. Circuit dynamics and neural coding in the locust olfactory system. In: *Encyclopedia of Neuroscience.* In press.

Jayaraman, V. and Laurent, G. Circuit dynamics and neural coding in the locust olfactory system. In: *Encyclopedia of Neuroscience.* In press.

Jayaraman, V. and Laurent, G. (2007) Evaluating a genetically encoded optical sensor of neural activity using electrophysiology in intact adult fruit flies. *Front. Neurosci.* In press.

Jortner, R.A., Farivar, S. and Laurent, G. (2007) A simple connectivity scheme for sparse coding in an olfactory system. *J. Neurosci.* **27**(7):1659-1669.

Jortner, R.A., Farivar, S. and Laurent, G. (2007) A simple connectivity scheme for sparse coding in an olfactory system. *J. Neurosci.* **27**(7):1659-1669.

Laurent G., Borst A. (2007) Dendritic short stories from invertebrates: linking biophysics and computation. In: *Dendrites*, Stuart G., Spruston N., Hauesser M., editors, 2nd Edition. Oxford University Press. In press.

Moreaux L, Laurent, G. (2007) Estimating firing rates from calcium signals in locust projection neurons *in vivo. Front. Neurosci.* In press.

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Summary: We continue our work on biophysical, neuroscience, and medical aspects of ion channels, receptors, and transporters. We have analyzed several strains of knock-in mice generated in our laboratory for the nicotinic α4 receptor, and these have resulted in interesting insights into nicotine addiction. We previously found that activation of α4-containing receptors by nicotine is sufficient for tolerance, sensitization, and reward behavior. The nicotinic receptor work is enhanced by a promising new knock-in strain, with fluorescent α4 receptors. We now report that chronic nicotine-produces cell type-specific increase in receptor levels, and these increases produce plausible explanations for some aspects of nicotine addiction.

These cell-specific increases in α4* receptors also provide a mechanism for the inverse correlation between smoking and Parkinson's disease. We are undertaking a systematic study of high-sensitivity nicotinic receptors in the basal ganglia, and of their regulation by exposure to chronic nicotine. A mouse model for Alzheimer's disease, another neurodegenerative disorder, is studied by Dr. Jankowsky in our group.

Because the α6 nicotinic subunit is expressed almost exclusively in dopaminergic neurons, it provides a promising therapeutic target. We have also generated BAC transgenic mice with hypersensitive α6 receptors. We are now characterizing the phenotype of the mice. This research on nicotine has been aided by a National Cooperative Drug Discovery Group, which is directed at Caltech and includes collaborators in at the University of Colorado, Boulder, and at Targacept.

The group's research has now been enhanced by two new microscopes: a Nikon spectrally-resolved confocal system, and TIRF capabilities for an existing wide field instrument. The TIRF capabilities are being extended to single molecule resolution.

The α4 nicotinic receptor, or its partner the β2 subunit, are mutated in a rare series of human epilepsies. We studied the nAChR epilepsy mutations in oocyte expression systems and, in collaboration with other labs, have reported knock-in mouse strains that also have these mutations.

Our work on selective silencing of mammalian neurons has generated a promising set of techniques and reagents-based on ligand-activated chloride channels. In collaboration with the David Anderson laboratory, we reported experiments on "proof of concept" silencing in mice. Now we are attempting to use the silencing vectors as reagents for "pharmacological deep brain stimulation" in mouse models for Parkinson's disease and epilepsy. We are also generating cation-permeable versions of the channels.

We continue our joint work with the Dougherty group, in Caltech's Chemistry Division, on aspects of ion channel structure-function. We have brought novel techniques to these studies, including mass spectrometry and fluorescence. We also concentrate on unnatural amino-acid mutagenesis. We have now found that a cation-π interaction helps agonists bind to several cys-loop receptors—for acetylcholine (now including neuronal receptors), serotonin, and GABA. By employing a new TIRF microscope, we are measuring individual nicotinic receptors containing unnatural florescent side chains.

With the Dougherty group and with Neurion Pharmaceuticals in Pasadena, we have started to analyze the binding of blocking drugs to the hERG potassium channel, also using unnatural amino acid mutagenesis. The channel is important because it underlies many instances of drug-induced long-QT syndrome. We are working hard to incorporate unnatural amino acids into the hERG channel when expressed in mammalian cells, where the pharmacology of blockade is much more similar to that in heart muscle.

Our work continues on quantitative aspects of transporter function, primarily measured with fluorescence. We are using fluorescence to analyze the

mobility of GABA transporters, and both confocal and TIRF modes.

Our group's home page has additional up-to-date information, images, and notices of positions. It's at http://www.cco.caltech.edu/~lester.

262. The role of cation-π interactions in nicotinic receptor activation

Jai A.P. Shanata[*], Bruce N. Cohen, Dennis A. Dougherty[*], Henry A. Lester

Our previous results show that a cation-π interaction between the agonist acetylcholine (ACh) and a tryptophan residue (αW149) in the extracellular domain (ECD) of the muscle nicotinic acetylcholine receptor (nAChR) dramatically enhances the ability of ACh to activate this receptor. To activate a receptor, the agonist must first bind to it and then induce conformational change(s) leading to activation. To determine which of these steps the cation-π interaction at residue αW149 primarily affects, we expressed wild type (wt) and mono-fluorinated mutant (5-F-W149) nAChRs in *Xenopus* oocytes and recorded their single-channel activity in cell-attached patches with 300 μM ACh. We previously found that modifying the cation-π interaction at residue αW149 by fluorinating this residue (5-F-W149) reduces the ACh sensitivity (i.e., increases the EC_{50}) of the muscle nAChR six-fold. Analyses of our single-channel kinetic data show this mutation reduced the ACh gating equilibrium constant (θ) less than two-fold, far short of the 36-fold decrease required to account for the six-fold increase in EC_{50} ($EC_{50} \sim\theta^{-1/2}$). In contrast, fitting the wt and 5-F-W149 mutant single-channel data to a four-state kinetic model with three agonist-bound closed states suggests that the 5-F-W149 mutant reduced the apparent forward rate constant for ACh binding seven-fold, enough to account for the effects of tryptophan fluorination on the EC_{50}, but this rate constant could also reflect rapid desensitization of the receptor rather than agonist binding.

Our experiments using ACh did not allow resolution of the fastest rate constant (channel opening). Two different methods will be employed in order to verify that the binding rate, as opposed to θ, is being diminished by fluorination. (1) The partial agonists tetramethylammonium and choline, which are known to have channel opening rates that are approximately 20 and 1000 times slower than ACh, respectively, will be used in order to directly measure the channel opening rate. (2) Patches will be obtained at multiple ACh concentrations in order to estimate the opening rate from the effective opening rate.

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263. Examining the relationship between γW55/δW57 and pore mutations in the nAChR

Kristin R. Gleitsman[*], Jai A.P. Shanata[*], Shawna Frazier, Henry A. Lester, Dennis A. Dougherty[*]

The muscle nicotinic acetylcholine receptor (nAChR) is a pentameric ligand gated ion channel (LGIC) with stoichiometry $\alpha_2\beta\gamma\delta$. Using two-electrode voltage clamp, we have discovered several sites in the extracellular domain (ECD) of the muscle nAChR which exhibit energetic coupling to the pore domain, specifically to a mutation termed βL9'S. Introduction of the βL9'S mutation into wild-type (wt) muscle nAChRs results in a gain-in-function mutant with an EC_{50} that is 40-fold lower (1.2 μM versus 50 μM for wt)—an effect that has been attributed to a significant increase in open channel lifetime. From the whole-cell data alone, the energetic coupling between the ECD and channel pore manifests itself as a decrease in the extent to which βL9'S causes gain-in-function. Thus, we are now performing single-channel recording (in the cell-attached configuration) using acetylcholine (ACh) at EC_{50} on αβγW55YδW57Y and αβL9'SγW55YδW57Y in order to test the hypothesis that the energetic coupling observed in some mutants (such as αβγW55YδW57Y) operates via a relatively smaller increase in open-channel lifetime upon addition of βL9'S.

Another mutation that we are exploring involves the introduction of α-hydroxy tryptophan (Wah) in the complementary subunits of the muscle nAChR binding site. α-hydroxy analogues of amino acids, in which the backbone amide bond is replaced with an ester, disrupt hydrogen bonding and introduce additional backbone flexibility, while conserving the side chain. This Wah mutation at certain conserved aromatic residues in the binding site, when coupled with βL9'S (αβL9'SγW55WahδW57Wah), yields a dose-response curve comprised of two components. In order to further explore the cause of this biphasic behavior and to develop a model of the states of the receptor corresponding to the individual components of the biphasic dose-response curve, we are performing single-channel recording (in the cell-attached configuration) at multiple concentrations on this mutant.

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264. Improved amber and opal suppressor tRNAs for incorporation of unnatural amino acids *in vivo*, Part 1: Minimizing misacylation

Erik A. Rodriguez[*], Dennis A. Dougherty[*]

The incorporation of unnatural amino acids (UAAs) site-specifically is a valuable technique for structure-function studies, incorporation of biophysical probes, and determining protein-protein interactions. THG73 is an amber suppressor tRNA used extensively for the incorporation of greater than 100 different residues in over 20 proteins, but under certain conditions THG73 is aminoacylated *in vivo* by endogenous aminoacyl-tRNA synthetase (aaRS). Similar aminoacylation is seen with the

E. coli Asn amber suppressor (ENAS) tRNA, which has also been used to incorporate UAAs in many studies. We now find that the natural amino acid (aa) placed on THG73 is Gln. Since the *E. coli* GlnRS recognizes positions in the acceptor stem, we made several acceptor stem mutations in the 2nd to 4th positions on THG73. All mutations reduce aminoacylation *in vivo* and allow for the selection of highly orthogonal tRNAs. To show the generality of these mutations, we created opal suppressor tRNAs that show less aminoacylation in *Xenopus* oocytes relative to THG73. We have created a library of *T. thermophila* Gln amber suppressor (TQAS) tRNAs that will be useful for determining optimal suppressor tRNAs for use in other eukaryotic cells.

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265. Improved amber and opal suppressor tRNAs for incorporation of unnatural amino acids *in vivo*, Part 2: Evaluating suppression efficiency

Erik A. Rodriguez[*], *Dennis A. Dougherty*[*]

The incorporation of unnatural amino acids (UAAs) into proteins is a valuable tool for addition of biophysical probes, bioorthogonal functionalities, and photo-reactive cross-linking agents, although these approaches often require quantities of protein that are difficult to access with chemically aminoacylated tRNAs. THG73 is an amber suppressor tRNA that has been used extensively, incorporating over 100 residues in 20 proteins. *In vitro* studies have shown that the *E. coli* Asn amber suppressor (ENAS) suppresses better then THG73. However, we report here that ENAS suppresses with less than 26% of the efficiency of THG73 in *Xenopus* oocytes. We then tested the newly developed *T. thermophila* Gln amber suppressor (TQAS) tRNA library, which contain mutations in the 2nd to 4th positions of the acceptor stem. The acceptor stem mutations have no adverse effect on suppression efficiency, and in fact can increase the suppression efficiency. Combining mutations causes an averaging of suppression efficiency, and increased suppression efficiency does not correlate with increased ΔG of the acceptor stem. We created a *T. thermophila* opal suppressor, TQOpS', which shows ≈50% suppression efficiency relative to THG73. The TQAS tRNA library, composed of functional suppressor tRNAs, has been created and will allow for screening in eukaryotic cells, where rapid analysis of large libraries is not feasible.

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266. Probing the muscarinic Type-2 acetylcholine receptor binding site through incorporation of unnatural amino acids

Michael M. Torrice[*], *Dennis A. Dougherty*[*]

The G-protein-coupled receptor (GPCR) family of transmembrane proteins is a target for a large number of pharmaceuticals on the market. GPCR signaling pathways are involved in vision, analgesia, drug addiction, memory, and the regulation of heartbeat and bronchial constriction. The muscarinic Type-2 acetylcholine receptor (M_2AChR) belongs to the aminergic class of family A GPCRs. Previous work in this group on the nicotinic acetylcholine receptor has shown that the cationic acetylcholine (ACh) was bound through a cation-π interaction with a conserved Trp residue. Studies on the M_2AChR binding site will focus on understanding the roles of conserved anionic and aromatic residues in binding cationic amine ligands.

Incorporation of unnatural amino acids into the M_2AChR will allow for chemical-scale studies of the receptor-binding site. A series of fluorinated aromatic amino acids will be incorporated at W3.28, W6.48 Y6.51, and W7.40 to probe the existence of a cation-π interaction. The unnatural amino acid, nitrohomoalanine (Nha), will be incorporated at D3.32 to probe electrostatic interactions between receptor and ligand, as suggested in the literature. Nha is isosteric to Asp, but lacks a net negative charge. Preliminary studies incorporating 5, 7-difluoro Trp (F_2W) at the conserved aromatic residues, W6.48 and W7.40, have shown that W7.40 is the possible site of a cation-π interaction between M_2AChR and ACh.

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267. Nicotine in the CNS vs. the neuromuscular junction: A cation-π interaction provides a crucial discrimination

Xinan (Joanne) Xiu[*], *Nyssa Puskar*[*], *Dennis Dougherty*[*]

Targeting specific nicotinic receptors has been proposed as a new therapeutic strategy for Alzheimer's disease, schizophrenia, Parkinson's disease, pain, ADHD, epilepsy, depression, smoking cessation and etc. Small molecules designed as the agonists, antagonists or modulators of these receptors have shown tremendous clinical potentials and several of them are in different stages of clinical trials at this moment.

Nicotinic receptors are a large family of ligand-gated ion channels, and at least 17 genes have been identified to code for different subunits of these channels. Nicotinic receptors are distributed widely throughout the central and peripheral systems. In the CNS, the two major nicotinic receptors are the neuronal type $\alpha4\beta2$ and the $\alpha7$ receptors, which account for more than 90 percent of high affinity nicotine binding and most of alpha-bungarotixin binding in the brain. At the neuromuscular junctions in the PNS, muscle receptors mediate the fast electric signal transmission that generates the muscular contractions.

As the prototypic nAChR, the muscle receptor received extensive pharmacological studies. Acetylcholine and epibatidine, but not nicotine, bind the muscle receptor through cation-π interactions with an important tryptophan (TrpB) at the ligand-binding site. The neuronal nAChR, presenting a much higher responsiveness to nicotine than the muscle type, are less studied.

Here we describe the binding of ligands to $\alpha4\beta2$ and $\alpha7$ using unnatural amino acid incorporation by

nonsense suppression methodology. A strong cation-π interaction between nicotine and α4β2 reveals the mechanism of nicotine binding in the CNS, providing a molecular basis for the high vs. low affinity of nicotine in the central vs. peripheral nervous systems. Distinctly different ligand binding modes in the two major CNS neuronal receptors offers insight into the design of subtype specific small molecules.

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268. Mutagenesis studies of conserved Phe and Pro residues in the Cys-loop of the mouse muscle nAChR

Walrati Limapichat[*], Dennis A. Dougherty[*]

Nicotinic acetylcholine receptors are prototypic members of the Cys-loop ligand-gated ion channels. Every subunit of the receptors in the family contains a Cys-loop, a signature sequence of thirteen residues flanked by cysteines bonding covalently through a disulfide bond to form a closed loop. At the apex of the Cys-loop is the sequence Phe-Pro, which is universally conserved throughout the family. The recent crystal structure (PDB ID 2qc1) showed that the proline existed in the *cis*-form, and the Phe and Pro side chains are stacked, making Van der Waals contact. Whether this Cys-loop proline undergoes *cis-trans* isomerization during the channel gating is the primary focus of our investigation. To this end, unnatural proline derivatives were incorporated at this site in the α_1 subunit of the mouse muscle nAChR. Preliminary data suggested that even a slight structural change could alter the receptor's sensitivity toward the agonist acetylcholine compared to the wild-type receptor. When unnatural derivatives of Phe were incorporated at the Phe site, the resulting mutant receptors were hypersensitive toward acetylcholine. Interestingly, these mutations also resulted in an increase in the relative efficacy of the partial agonist succinylcholine compared to acetylcholine. Aromatic-proline interaction is known to stabilize the *cis*-proline isomer [Pal *et. al.* (1999) *J. Mol. Biol*. **294**:271-288], and thus, double mutagenesis studies were performed in order to investigate the Phe-Pro side-chain interaction. Removal of Phe aromaticity, either by alanine or cyclohexylalanine mutation, diminished the effect of unnatural mutations at the Pro residue. These data confirmed the presence of aromatic-proline interaction in the Cys-loop.

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269. Imaging the microorganization of synaptic receptors

Lawrence A. Wade, Daniel Lo[1], Scott Fraser[2]

Nicotinic acetylcholine receptors (nAChRs) are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR is a (pseudo)symmetric pentameric structure comprised of homologous subunits. Furthermore, the α subunits exist in at least ten different subtypes (α1 through α10) and the β subunits exist in at least four subtypes (β1 through β4). We're attempting to combine several novel techniques to directly image the microorganization of AChRs expressed in *Xenopus* oocytes. By expressing different color XFPs in AChR α and β subunits, we expect to resolve individual receptors and thereby determine the number of α and β subunits comprising them. Furthermore we hope to discretely identify individual receptors over a field-of-view of at least one micron. This will enable us to simultaneously characterize the large-scale distribution of such receptors, to identify any local structures within an imaged membrane surface, and to assay variation of receptor subtypes. The techniques being combined in this experiment include a novel method for repeatedly locating imaging probes to specific substrate locations within a few 10's of nm, and an ~10 nm resolution, single-molecule sensitive near-field optical microscope. A technique for attachment protein patterning on glass coverslips via dip-pen nanolithography (DPN) will be used to adhere the membranes to a glass substrate. Receptor subunit imaging is enabled by the development of specific XFP-labeled AChR subunits. By developing the ability to directly image each subunit of each AChR expressed in the membrane of *Xenopus* oocytes, we lay the groundwork for future studies of transmembrane proteins, receptors and membrane-embedded proteins in many different types of cells. In particular we will substantially advance our ability to image the microorganization of nicotinic acetylcholine receptors at neuronal synapses.

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270. Detection of a single fluorescent unnatural amino acid incorporated into the nAChR

Rigo Pantoja, Erik A. Rodriguez[*], Mohammed I. Dibas, James E. Petersson[*], E. James Petersson[*], Dennis A. Dougherty[*]

Fluorescent unnatural amino acids (fUAAs) represent an attractive strategy for labeling ion channel proteins. Here we report the first successful incorporation of a fUAA, Lys(BODIPYFL), into the muscle nicotinic acetylcholine receptor (nAChR). *Xenopus* oocytes were injected with the frameshift suppressor tRNA aminoacylated with Lys(BODIPYFL) (YFaFSACCC-Lys(BODIPYFL)) and nAChR (α/β19'GGGU/δ/γ) mRNAs. Two-electrode voltage-clamp recordings confirmed the presence of functional surface-expressed nAChRs with an ACh EC50 of 38 ± 2 μM and Hill coefficient of 1.12 ± 0.05, respectively (n = 5). We measured fluorescence from oocytes expressing the nAChR β19'GGGULys(BODIPYFL) using single molecule total internal reflection fluorescence (TIRF) microscopy. Under conditions of relatively low expression (<0.25 receptors/μm^2), we resolved discrete puncta; time-series photobleaching experiments determined that 70% of observed puncta originated from single molecules since a discrete decrease in fluorescence resulted upon photobleaching. Puncta displayed a Gaussian distribution

of intensities; the average single-molecule signal to background (ΔF/F) ± standard deviation (SD) was 0.23 ± 0.01. Puncta were also detected in oocytes injected with only YFaFSACCC-Lys(BODIPYFL), however at lower densities (~ 0.007 puncta per μm^2). To confirm that the puncta originated from Lys(BODIPYFL) incorporated into a nAChR, we incubated oocytes with α-Bungarotoxin mono-conjugated with Alexa488 (αBtxAlexa488). The nAChR β19'GGGULys(BODIPYFL) has two α-Btx binding sites; thus, three days after injection, labeled with αBtxAlexa488 displayed puncta with 1 (38%), 2 (34%), 3 (23%), and 4-6 (5%) discrete photobleaching steps (n = 2 oocytes, 3 image frames, 332 puncta). We also performed experiments with a nAChR mutant that contained eGFP in the γ subunit M3-M4 loop; as expected, these puncta were less intense than the BODIPYFL (ΔF/F ± SD ~ 0.14 ± 0.04). In addition, the nAChR γ_{eGFP} labeled with αBtxAlexa488 resulted in a similar distribution of 1 (54%), 2 (21%), 3 (20%) and greater than 4 (5%) photobleaching steps (n = 2 oocytes, 2 image frames, 265 puncta). The wild-type muscle nAChR labeled with αBtxAlexa488 resulted in 1 (77%), 2 (19%), and 3 (4%) photobleaching steps (n = 2 oocytes, 3 image frames, 306 puncta). Thus, we have demonstrated that Lys(BODIPYFL) can be incorporated into the nAChR and detected at the single molecule level. We also resolved single molecules on nAChRs with two conventional complimentary labeling schemes. The three labeling schemes were consistent with each other based on previous studies of the nAChRs.

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271. A modified structural model of the 5-HT_3 receptor in the region of loop A resulting from binding and functional studies

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Homology models of Cys-loop receptors require validation, especially in regions that have ambiguous sequence alignments such as binding loop A. We have used mutagenesis (with both unnatural and natural amino acids), radioligand binding, voltage clamp electrophysiology and homology modeling to probe the role of two critical adjacent residues in loop A of the 5-HT_3 receptor: Asn128 and Glu129. The data show that incorporation of a range of alternative amino acids at position 128 changes the EC_{50} from ~10 fold more potent than wild type to ~10 fold less potent, and also changes the maximal peak current for *m*CPBG compared to that for 5-HT (R_{max}) up to 19 fold. Modifications at position 129 result in functional receptors only when the residue is able to accept a hydrogen bond, and these decrease R_{max} by up to four fold. Partial agonists are unable to activate some Glu129 mutant receptors but simply inhibit their function with an IC_{50} similar to their wild-type EC_{50}. Overall the data support the hypothesis that Asn128 has a role in facilitating transitions between conformational states but Glu129 participates primarily in ligand binding, to a lesser extent in gating, and also influences receptor expression. In the resulting modified model of the 5-HT_3 receptor extracellular domain, Glu129 and not Asn128 faces into the binding pocket.

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272. Conformational mobility of the glycine receptor M2 domain during activation, antagonism and allosteric modulation

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We investigated ligand-induced conformational changes in the homomeric α1 glycine receptor M2 pore-lining domain. This was achieved by tethering a rhodamine fluorophore to a cysteine introduced at the 19' position near the extracellular M2 boundary, and monitoring fluorescence and current changes as the channel was opened and closed by different classes of ligands. During glycine activation, fluorescence increased by ~20% and the emission peak shifted to lower wavelengths, consistent with a more hydrophobic fluorophore environment. In contrast, the agonist ivermectin activated the receptors without producing a fluorescence change. Although taurine and β-alanine were weak partial agonists at the α1R19'C GlyR, they induced large fluorescence changes. Propofol, which drastically enhanced these currents, did not induce a glycine-like blue-shift in the fluorescence spectral emission peak. These results show that different agonists can produce different M2 domain conformational changes. The inhibitors, strychnine and picrotoxin, elicited fluorescence and current changes as expected for a competitive antagonist and an open channel blocker, respectively. On the other hand, the putative channel blocker, ginkgolide C, produced an increase in fluorescence suggestive of a direct interaction between rhodamine and a low affinity ginkgolide site. Finally, our results suggest that rhodamine attached to a glycine-bound subunit responds differently to the same fluorophore attached to a glycine-free subunit, implying that activation is mediated by sequential rather than fully concerted transitions. Taken together, our results shed new light on the conformational flexibility of the GlyR M2 helix during activation, antagonism and allosteric modulation.

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273. Novel seizure phenotype and sleep disruptions in knock-in mice with hypersensitive α4* nicotinic receptors

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A leucine to alanine substitution (L9'A) was introduced in the M2 region of the mouse α4 neuronal nicotinic acetylcholine receptor (nAChR) subunit. Expressed in *Xenopus* oocytes, α4(L9'A)β2 nAChRs were ≥ 30-fold more sensitive to both ACh and nicotine. We generated knock-in mice with the L9'A mutation and studied their cellular responses, seizure phenotype, and sleep-wake cycle. Seizure studies on α4-mutated animals are relevant to epilepsy research because all known mutations linked to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) occur in the M2 region of α4 or β2 subunits. Thalamic cultures and synaptosomes from L9'A mice were hypersensitive to nicotine-induced ion flux. L9'A mice were ~15-fold more sensitive to seizures elicited by nicotine injection than their WT littermates. Seizures in L9'A mice differed qualitatively from those in WT: L9'A seizures started earlier, were prevented by nicotine pretreatment, lacked EEG spike-wave discharges and consisted of fast repetitive movements. Nicotine-induced seizures in L9'A mice were partial whereas WT seizures were generalized. When L9'A homozygous mice received a 10 mg/kg nicotine injection, there was temporal and phenomenological separation of mutant and WT-like seizures: an initial seizure, ~20 s after injection was clonic and showed no EEG changes. A second seizure began 3-4 min after injection, was tonic-clonic and had EEG spike-wave activity. No spontaneous seizures were detected in L9'A mice during chronic video-EEG recordings, but their sleep-wake cycle was altered. Our findings show that hypersensitive α4* nicotinic receptors in mice mediate changes in the sleep-wake cycle and nicotine-induced seizures resembling ADNFLE.

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274. Uncoupling benzodiazepine potentiation of GABA(A)R

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The γ-amino butyric (GABA) type A receptor ($GABA_AR$) is an inhibitory ion channel in the cys-loop ligand gated ion channel superfamily. Like all other members of this family, the GABA(A)Rs are comprised of five homologous subunits arranged pseudo-symmetrically around a central ion pore. Although there are a large number of GABA(A)R subunits, we focus on the most prevalent type which consists of two copies of each of the α_1 and β_2 subunits as well as one copy of the γ_2 subunit, arranged as β–α–β–α–γ. GABA binding sites are located at each β/α interface. Although the β subunit is considered the principal subunit for ligand binding, the α subunit contributes residues necessary for proper ligand binding and channel function as well. The α subunit is the principal subunit involved in benzodiazepine (BZD) binding, located at the α/γ binding site. Binding of benzodiazpines to this site is known to potentiate the GABA-induced current of these receptors. Although the potentiation is well documented, the mechanism is unknown and is the current focus of this project. We propose to probe the involvement of a beta-strand in the α subunit for involvement in the mechanism of benzodiazepine potentiation. Our interest in this beta-strand is based on physical proximity as it extends from R119 and I120, residues implicated in GABA binding, to H101, a residue implicated in BZD binding.

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275. Evaluating interfacial residues of the NMDA receptor with unnatural amino acids: A novel probe of the clamshell

Kathryn A. McMenimen[*], Dennis A. Dougherty[*]

Ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels that play a central role in learning and memory in the mammalian central nervous system. The N-methyl-D-aspartate (NMDA) receptor is activated by the binding of glutamate and glycine to a clamshell-like domain in the NR2 and NR1 subunits. Ligand binding rotates the D2 lobe of the clamshell toward the D1 lobe, and this closure promotes channel gating. Studies of partial agonists in the related AMPA receptor establish that submaximal activation results from partial clamshell closure. However, structural studies of NR1 have not yet revealed a similar mechanism for partial agonist activation. Using unnatural amino acid mutagenesis we incorporate homotyrosine, lengthening an NR2 conserved tyrosine by a single CH_2. Homotyrosine incorporation has two correlated effects for a series of four partial or full agonists: the mutation disrupts clamshell closure and also produces larger functional changes for full agonists relative to partial agonists. This data shows that a clamshell closure mechanisms, previously shown for AMPA receptors, probably also apply to NMDA receptors. This study extends the unnatural amino methodology to serve as a functional probe of the clamshell mechanism in iGluR ligand binding.

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276. Incorporating *p*-amino-Phe into proteins through nonsense suppression

Amy L. Eastwood[*], Dennis A. Dougherty[*]

Cationic drugs can bind to tryptophan, tyrosine, or phenylalanine residues in an ion channel through a cation-π interaction [one example, Zhong *et al.* (1998) *PNAS* **95**:12088]. Here, the face of the aromatic residue stabilizes the cation through a predominantly electrostatic interaction. Computational calculations using a sodium

ion as the cation found that phenylalanine and tyrosine have about equal binding energies, while tryptophan has a higher binding energy than both and therefore binds cations more tightly [Mecozzi *et al*. (1996) *PNAS* **93**:10566]. The unnatural amino acid *p*-amino-Phe was found to have a similar binding energy to tryptophan, leading us to wonder whether tryptophan is nature's way of creating a non-oxidizable *p*-amino-Phe.

In an effort to test whether *p*-amino-Phe can increase the affinity of a drug for a receptor, this unnatural amino acid was synthesized and is being incorporated into the binding sites of various receptors with tyrosine- and phenylalanine-containing binding sites, such as the $GABA_A$ receptor, the $GABA_C$ receptor, and the Shaker K^+ channel. *p*-amino-Phe has given varying results in each channel in which its been successfully incorporated, and in no case has it enhanced the cation-π interaction. Systematic tests have not uncovered why the results differ from the predictions. In an effort to test whether the oocyte is altering the aniline, the unnatural amino acids *p*-nitro-Phe and *p*-acetamido-Phe were synthesized. In the case of the Shaker K^+ channel, *p*-amino-Phe and *p*-acetamido-Phe gave the same IC_{50} for the cationic blocker TEA but further work will need to be done to confirm that *p*-amino-Phe is indeed being converted into *p*-acetamido-Phe inside the oocyte.

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277. Nicotine responses in hypersensitive and knockout α4 mice account for tolerance to both hypothermia and locomotor suppression in wild-type mice

Andrew R. Tapper[1], Sheri L. McKinney, Michael J. Marks[2]

The mechanistic contributions of alpha4* nAChRs in behavioral tolerance, a hallmark of nicotine dependence, are unclear. To evaluate the contribution of alpha4* nAChRs and non-alpha4 nAChRs in the development of tolerance to hypothermia and locomotor suppression, alpha4 knockout (KO) and hypersensitive Leu9'Ala alpha4 knock-in mice were injected with nicotine and their behaviors were compared to nicotinic responses in wild-type (WT) mice. Repeated selective activation of alpha4* nAChRs in Leu9'Ala mice produced profound tolerance to hypothermia over seven days, whereas no tolerance was observed in alpha4 KO animals. The summed time course and temperature response (after appropriate normalizations) from these two mutant mouse strains was not significantly different from the time course of WT tolerance. In addition, daily selective activation of alpha4* nAChRs elicited locomotor activation in Leu9'Ala mice, but nicotine suppressed activity in alpha4 KO mice and this did not change with daily drug exposure. Again, appropriately combined responses from the two mutant strains reflected the biphasic nicotine-induced activity in WT animals. These data indicate that, by analyzing nicotinic responses in two complementary mouse lines, one lacking alpha4* nAChRs, the other expressing hypersensitive alpha4* nAChRs, one can accurately separate non-α4 nAChR responses from alpha4 nAChR responses, and one can also account for WT tolerance to both hypothermia and locomotor suppression. Together our data suggest a new paradigm for bridging the gap between genetic manipulation of a single receptor and whole-animal behavioral studies, and show that activation of alpha4* nAChRs is both necessary and sufficient for the expression of tolerance.

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278. Nicotine induces tonic seizures in an ADNFLE mouse

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Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is a type of inherited partial epilepsy that is linked to several different point mutations in the α4 and β2 nicotinic subunits. In humans, these mutations produce brief (1-5 min) nocturnal seizures that can include tonic extension of the neck, trunk, and/or forelimbs; tonic finger spreading; and forelimb clonus at the end of the seizures. Activation of the mutant receptors by an abnormal increase in acetylcholine (the endogenous agonist) release is thought to trigger the seizures. Our collaborator (Jian Xu) at the Salk Institute constructed a knock-in mouse line bearing one of the ADNFLE mutations (β2:V287L). To determine whether nicotine-induced ADNFLE-like seizures in these mice, we injected the mutant mice and their wild-type (WT) littermates with 0.5-8 mg/Kg nicotine (i. p.) and observed their behavior. Interestingly, low doses of nicotine (0.5-2 mg/Kg) elicited a strong Straub tail response (tail erection) accompanied by tonic trunk and neck extension. In the 1-2 mg/Kg range, nicotine also elicited tonic forelimb extension accompanied by tonic spreading of the forepaw digits and occasional forelimb clonus. Nicotine-induced tonic limb extension and digit spreading appeared to be unique to the mutant mice. A slightly higher nicotine dose (3 mg/Kg) produced forelimb clonus in most mutant mice. In contrast, 0.5-3 mg/Kg nicotine produced only weak tail erection and sedation in the WT mice. Higher nicotine doses (4-8 mg/Kg) produced forelimb clonus and clonic convulsions in both the WT and mutant mice but the mutant mice were hypersensitive to the effects of nicotine in this range and the latencies to nicotine-induced clonus and convulsion were shorter. Thus, low nicotine doses (1-2 mg/Kg) elicit tonic seizures in the β2:V287L mice with behavioral features resembling those observed in human ADNFLE patients.

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279. Nicotine-induced dystonic arousal complex in a mouse line harboring a human autosomal dominant nocturnal frontal lobe epilepsy mutation

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We generated a mouse line harboring an autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) mutation: the α4 nicotinic receptor S248F knockin strain. In this mouse, modest nicotine doses (1-2 mg/kg) elicit a novel behavior termed the dystonic arousal complex (DAC). The DAC includes stereotypical head movements, body jerking, and forelimb dystonia; these behaviors resemble some core features of ADNFLE. A marked Straub tail is an additional component of the DAC. Similar to attacks in ADNFLE, the DAC can be partially suppressed by the sodium channel blocker carbamazepine or by pre-exposure to a very low dose of nicotine (0.1 mg/kg). The DAC is centrally mediated, genetically highly penetrant, and, surprisingly, not associated with overt ictal electrical activity as assessed by: (1) epidural or frontal lobe depth electrode electroencephalography; or (2) hippocampal c-fos regulated gene expression. Heterozygous knockin mice are partially protected from nicotine-induced seizures. The noncompetitive antagonist mecamylamine does not suppress the DAC, even though it suppresses high dose nicotine-induced wild-type-like seizures. Experiments on agonist-induced $^{86}Rb^{+}$ and neurotransmitter efflux from synaptosomes and on α4S248Fβ2 receptors expressed in oocytes confirm that the S248F mutation confers resistance to mecamylamine blockade. Genetic background, gender, and mutant gene expression levels modulate expression of the DAC phenotype in mice. The S248F mouse thus appears to provide a model for the paroxysmal dystonic element of ADNFLE semiology. Our model complements what is seen in other ADNFLE animal models. Together these mice cover the spectrum of behavioral and electrographic events seen in the human condition.

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280. Cell autonomy, receptor autonomy, and thermodynamics in nicotine receptor up-regulation

Raad Nashmi, Henry Lester

Chronic nicotine exposure, in smokers or in experimental rodents administered nicotine, produces elevated levels of nicotinic acetylcholine receptors in several brain regions. However, there are few data on up-regulation of receptors in specific neuronal subtypes. We tested whether functional up-regulation of nicotinic responses occurs in cultured GABAergic neurons of the ventral midbrain. Fura-2 measurements of nicotinic responses were made on ventral midbrain neurons from knock-in mice heterozygous for the α4-M2 domain Leu9'Ala mutation, which confers nicotine hypersensitivity. Chronic nicotine exposure at a concentration (10 nM for three days) that activates only the hypersensitive α4*(Leu 9' Ala) receptors, but not wild-type receptors, resulted in significant potentiation of ACh (100 μM)-elicited responses. Experiments were also performed on midbrain neuronal cultures heterozygous for the α4*(Leu9'Ala) mutation as well as for a GFP protein fused to a GABA transporter that reliably reveals GABAergic neurons. In cultures chronically treated with 10 nM nicotine, there was significantly increased α4* nicotinic-induced Ca^{2+} influx elicited by low concentration of ACh (3 μM). Furthermore, chronic exposure to the competitive antagonist dihydro-β-erythroidine, but not to the noncompetitive antagonist mecamylamine, induced up-regulation of ACh elicited nicotinic responses. These results suggest that occupation of α4* nicotinic receptor binding site(s), at the interface between two subunits, is sufficient to promote assembly and/or up-regulation of functional receptors in GABAergic neurons. Up-regulation in neurons is both, "cell-autonomous," occurring at the cell itself, and "receptor autonomous," occurring at the receptor itself, and may be a thermodynamic necessity of ligand-protein interactions.

281. Photons from cyan and yellow fluorescence proteins shed light on α4β2 nAChR subunit assembly and stoichiometry

Cagdas D. Son, Raad Nashmi, Larry Wade, Ryan Drenan

Although we know that neuronal nicotinic acetylcholine receptors (nAChRs) are the primary target for nicotine, the mechanisms that cause nicotine addiction are poorly understood at the molecular, cellular, and circuit level. Therefore, an improved overall understanding of addiction and of nicotine as an addictive drug is crucial in developing medications and behavioral treatments for tobacco addiction. The α4β2 nicotinic acetylcholine receptor (nAChR) is the most abundant nAChR expressed in mammalian brain, where it forms a high-affinity binding site for nicotine. α4 and β2 nAChR subunits expressed heterologously assemble into receptors with high and low sensitivity to acetylcholine (ACh). The relative proportions of these two receptor populations depend on

the α4 to β2 ratio and can be altered by chronic nicotine exposure. In this study, we used normally functioning fluorescently-tagged α4 and β2 subunits to identify receptor assembly and subunit stoichiometry by quantitative Förster resonance energy transfer (FRET) measurements as well as by florescence intensity ratio (FIR) analysis in live cells.

a. Neuro2a cells were transfected with 1:9, 1:1 and 9:1 ratios of α4:β2 nAChR subunits to force receptors with differing agonist sensitivity (high, mixed, and low sensitivity, respectively). Measurements on α4YFP-β2CFP or α4CFP-β2YFP receptors showed that chronic nicotine increases the fraction of $(\alpha4)_2(\beta2)_3$ receptors by ~1.5. Previously, we, and others, found that chronic nicotine exposure augments assembled receptors by a similar fraction (Nashmi *et al.*, 2003).
b. Because FRET between neighboring identical subunits is large, and because a pentamer with three identical subunits has one such neighbor, FRET efficiencies for α4XFP-β2 or α4-β2XFP (XFP = 1:1 YFP : CFP) transfected cells also reveal subunit stoichiometry. The observed FRET agreed with expectations from: (1) forced stoichiometry; and (2) from chronic nicotine-induced increases in the fraction of $(\alpha4)_2(\beta2)_3$ receptors.
c. Availability of knock-in mice with fluorescently-tagged nAChR subunits enables similar, but technically challenging, experiments on primary cell cultures from relevant brain regions in the control and nicotine incubated preparations. Correlating the optical measurements from quantitative FRET and FIR analysis with dose-response data generated from functional measurements on the receptors themselves will be useful to understand cell specific regulation of α4β2 nAChRs upon chronic nicotine exposure.

282. Establishing an ion pair interaction in the $GABA_c$ receptor that contributes to the gating pathway

Jinti Wang[*]*, Dennis A. Dougherty*[*]

$GABA_C$ receptors are members of the Cys-loop superfamily of ligand-gated ion channels. Upon agonist binding, the receptor undergoes a structural transition from the closed to the open state, but the mechanism of gating is not well understood. Here we utilized a combination of conventional mutagenesis and the high precision methodology of unnatural amino acid incorporation to study the gating interface of the human homopentameric $GABA_C$ ρ1 receptor. We have identified an ion pair interaction between two conserved charged residues, E92 in loop 2 of the extracellular domain and R258 in pre-M1 region. We hypothesize that the salt bridge exists in the closed state by kinetic measurements and free energy analysis. Several other charged residues at the gating interface are not critical to receptor function, supporting previous conclusions that it is the global charge pattern of the gating interface that controls receptor function in the Cys-loop superfamily.

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283. α4* nicotinic receptors in the preBötzinger complex mediate cholinergic/nicotinic modulation of respiratory rhythm

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Acetylcholine and nicotine modulate respiratory patterns by acting on nicotinic acetylcholine receptors (nAChRs) in the preBötzinger Complex (preBötC). To further explore the molecular composition of these nAChRs, we studied a knock-in mouse strain with a leucine to alanine mutation in the M2 pore-lining region (L9'A) of the α4 subunits; this mutation renders α4-containing receptors hypersensitive to agonists. We recorded respiratory rhythm from hypoglossal nerve (XIIn) and whole-cell patch-clamped preBötC inspiratory neurons in a medullary slice preparation from neonatal mice *in vitro*. Nicotine affected respiratory rhythm at concentrations ~ 200-fold lower in the homozygous L9'A knock-in mice compared with wild-type mice. Bath application of just 5 nM nicotine increased respiratory frequency, induced tonic/seizure-like activities in XIIn, and induced responses in preBötC inspiratory neurons in the L9'A mice. Microinjection of nicotine into the preBötC increased respiratory frequency while injection into the ipsilateral hypoglossal (XII) nucleus induced tonic/seizure-like activity in the mutant mice. The α4β2*-selective nAChR antagonist dihydro-β-erythroidine (DHβE) produced opposite effects and blocked the nicotine responses. These data, showing that the nAChRs in the preBötC and XII nucleus in the L9'A mice are hypersensitive to nicotine and endogenous ACh, suggest that functional α4* nAChRs are present in the preBötC and mediate cholinergic/nicotinic modulation of the excitability of preBötC inspiratory neurons and regulation of respiratory rhythm. Furthermore, functional α4* nAChRs are present in XII nucleus and mediate cholinergic/nicotinic modulation of tonic activity in XIIn.

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284. Subcellular trafficking, pentameric assembly and subunit stoichiometry of neuronal nicotinic ACh receptors containing fluorescently labeled α6 and β3 subunits

Ryan M. Drenan, Raad Nashmi, Princess Imoukhuede, Herwig Just, Sheri McKinney, Henry A. Lester

Neuronal nicotinic ACh receptors are ligand-gated, cation-selective ion channels. Nicotinic receptors containing α4, α6, β2, and β3 subunits are expressed in midbrain dopaminergic neurons and are

implicated in nicotine addiction. Here we have studied the cell biological and biophysical properties of receptors containing α6 and β3 subunits by utilizing fluorescent proteins fused in-frame within the M3-M4 intracellular loop. Receptors containing fluorescently-tagged β3 subunits were fully functional compared to receptors with untagged β3 subunits. We find that β3 and α6-containing receptors are highly expressed in neurons, and co-localize with co-expressed, fluorescent α4 and β2 subunits in neuronal soma and dendrites. Förster resonance energy transfer (FRET) reveals efficient, specific assembly of β3 and α6 into nicotinic receptor pentamers of various subunit compositions. Using FRET, we demonstrate directly that only a single β3 subunit is incorporated into nAChRs containing this subunit, whereas multiple subunit stoichiometries exist for α4 and α6-containing receptors. Finally, we demonstrate that nicotinic ACh receptors are localized in distinct microdomains at or near the plasma membrane using total internal reflection fluorescence (TIRF) microscopy. We suggest that neurons contain large, intracellular pools of assembled, functional nicotinic receptors, which may provide them with the ability to rapidly up-regulate nicotinic responses to endogenous ligands such as ACh, or to exogenous agents such as nicotine. Further, this report is the first to directly measure nAChR subunit stoichiometry using FRET and plasma membrane localization of α6 and β3-containing receptors using TIRF.

285. Bacterial artificial chromosome (BAC) transgenic mice expressing hypersensitive $\alpha6^{L9'S}$ nicotinic ACh receptors: Isolation and amplification of electrophysiological and behavioral responses

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Nicotinic receptor activation during smoking stimulates dopaminergic neurons in the ventral tegmental area (VTA) and substantia nigra (SN). α4* and α6* (* denotes "containing") are key players in nicotine-dependent stimulation of dopamine release. Here, we report the construction and characterization of a new mouse model with hypersensitive α6 nAChRs to study nicotine addiction. To amplify and isolate α6-specific responses, we utilized bacterial artificial chromosome (BAC) recombineering and transgenesis. First, a BAC containing the entire genomic *Chrna6* (α6 nAChR) sequence was obtained. Homologous recombination in bacteria was used to introduce a point mutation (Leu 280 to Ser, aka Leu9'Ser) that results in a gain-of-function allele and a leftward shift in the concentration-response relationship for receptor activation by ACh or nicotine. Further, we silenced the adjacent *Chrnb3* (β3 nAChR) gene by disrupting the start codon with an ampicillin selection cassette. *Chrna6*$^{L9'S}$ BAC DNA was microinjected into fertilized oocytes followed by oviduct transfer to a pseudopregnant mouse. Transgenic founders were identified and bred for germline transmission by crossing to C57BL/6 mice.

Chrna6$^{L9'S}$ BAC transgenic mice have grossly normal body weight, grooming behavior, and social interaction with cagemates. Several lines express transgene mRNA. To further analyze the localization and numbers of α6* receptors in brains of $\alpha6^{L9'S}$ and WT cagemates, we performed [^{125}I]-αCtxMII binding experiments. In these experiments, α6 receptor numbers are roughly equivalent in transgenic and non-transgenic littermates. Further, we have found no ectopic expression of α6 receptors in any of our lines. To determine whether transgenic mice expressed functional, hypersensitive α6* receptors, we recorded from VTA dopaminergic neurons in slice preparation. $\alpha6^{L9'S}$ mice exhibited robust nicotine-stimulated inward currents (166 + 14 pA) in response to 1 μM nicotine, which were entirely attributable to α6-based on their complete inhibition by αCtxMII. Neurons from WT mice exhibited little or no activation of nAChRs at 1 μM nicotine. $\alpha6^{L9'S}$ mice exhibit abnormal stereotypic locomotor behaviors in their home cage that we are beginning to characterize. *Chrna6*$^{L9'S}$ BAC transgenic mice may be useful in future studies to develop α6-selective compounds for the treatment of Parkinson's disease and for smoking cessation therapy.

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286. Screening nicotinic ligands for smoking cessation therapy using acute hypothermic responses in $\alpha4^{L9'A}$ knock-in mice

R.M. Drenan, S. McKinney, I. Goldflam, E.H. Anamudu, H.A. Lester

More than 550,000 Americans die each year from exposure to cigarette smoke. Smokers find it extraordinarily difficult to quit smoking largely due to the addictive properties of nicotine. Current smoking cessation therapies are marginally effective, and 52-week abstinence rates are low (~15%) among people who attempt to quit. To design and develop new smoking cessation therapies, we, along with our collaborators at Targacept Inc., and the University of Colorado, have begun screening novel, nicotine-like compounds for *in vitro* and *in vivo* activity at nicotinic ACh receptors. Our *in vivo* assay for nicotinic receptor activation is acute hypothermia in wild-type and homozygous $\alpha4^{L9'A}$ knock-in mice. L9'A mice express mutant α4* (*denotes "containing") nAChR receptors that are 50 to 100 times more sensitive to agonist, allowing one to selectively isolate and amplify downstream responses. Previously, we have demonstrated that selective activation of α4* receptors is sufficient to induce hypothermia in these mice. Because α4* receptors are candidate targets for smoking cessation therapy drugs, these mice provide an excellent *in vivo* assay for bioavailability and efficacy at the target receptor. Six mice of each genotype are subcutaneously

implanted with Mini-Mitter temperature/activity probes and allowed to recover from surgery for several days. Following several days of saline injections, mice are injected with a test compound, and temperature fluctuations are recorded. For compounds known to have agonist or partial agonist properties, each is injected alone. Compounds known to be antagonists are pre-injected with the test compound followed ten minutes later by challenge with nicotine (0.015 mg/kg for L9'A and 1.5 mg/kg for wild type). We have nearly completed initial screening, and will proceed with further development of promising lead compounds in the next year.

287. Reversible genetic suppression of neuronal excitability in freely-behaving mice by expression of a Cl^- channel gated by a systemically administered ligand

Walter Lerchner, Cheng Xiao, Raad Nashmi, Eric M. Slimko, Laurent van Trigt, David J. Anderson[*]

Several genetic strategies for inhibiting neuronal function in mice have been described, but no system that directly suppresses membrane excitability, and is triggered by a systemically administered drug, has been validated in awake behaving animals. We expressed unilaterally in mouse striatum a modified heteromeric ivermectin (IVM)-gated chloride channel from *C. elegans* (GluClαβ), systemically administered IVM, and then assessed amphetamine-induced rotational behavior. Rotation was observed as early as 4 hr after a single intraperitoneal IVM injection (10 mg/kg), reached maximal levels by 12 hr, and was almost fully reversed by four days. Multiple cycles of silencing and recovery could be performed in a single animal. In striatal slice preparations from GluClαβ-expressing animals, IVM rapidly suppressed spiking. The two-subunit GluCl/IVM system permits "intersectional" strategies designed to increase the cellular specificity of silencing in transgenic animals.

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288. Selective silencing of mammalian neurons in the ventral-tegmental area to study addiction behavior

Herwig Just

Traditionally, the function of specific neurons in the central nervous system has been studied by inactivating them with lesioning methods. We have established a technique to selectively and reversibly silence mammalian neurons *in vivo*. This will allow us to investigate their function in the context of an intact circuitry. We are heterologously expressing a glutamate-gated chloride channel (GluCl) from the nematode *C. elegans* in mammalian neurons. The GluCl channel consists of alpha and beta-subunits that assemble into a heteropentamer. We have modified the subunits for our purposes: They have been codon-optimized for enhanced mammalian expression, tagged with fluorescent proteins to allow for direct visualization and had the tyrosine at residue 182 of the GluCl beta-subunit mutated to a phenylalanine to eliminate glutamate responses. Activation of GluCl with the allosteric activator ivermectin (IVM) elicits a current that clamps the neurons to the resting potential of chloride, thus inhibiting action potentials ('silencing'). We are using adeno-associated virus encoding the modified GluCl channel subunits to drive high expression levels in the neurons. Virus is injected stereotaxically into the ventral-tegmental area (VTA) of mice. VTA is part of the mesocorticolimbic reward pathway. Cells in the VTA send projecting fibers to the nucleus accumbens and prefrontal cortex releasing dopamine (DA). Drugs of abuse induce release of dopamine in the nucleus accumbens. We are blocking neuronal activity of mice injected in the VTA by administering IVM. Mice are given drugs of abuse (cocaine, amphetamine, nicotine) and the block of the reward pathway is studied in place-preference assays.

289. Alternative approach to deep brain stimulation in Parkinson's disease using ion channels

Herwig Just, Sindhuja Kadambi

Parkinson's disease (PD) is a degenerative neurological disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta. This leads to altered neuronal activity within the basal ganglia. Pharmacological treatment with L-DOPA is effective at substituting for the loss of dopamine in the striatum during the in the early stages of the disease. Surgical approaches have been developed to treat those patients who fail to respond to pharmacological therapy. Along with surgical ablation of affected areas, deep brain stimulation (DBS) has become the preferred treatment for late stages of PD. A stimulating electrode is permanently implanted into basal ganglia structures. High frequency stimulation leads to improvement of motor symptoms (tremor, bradykineasia) in PD patients. The mechanisms underlying DBS have not been clarified; both inhibition and stimulation of neuronal activity by high-frequency simulation have been suggested. We are using an ion channel-based strategy to investigate the underlying mechanism and to develop an alternative approach to DBS. We have developed a technique to inhibit neuronal activity in mammalian cells by heterologously expressing a glutamate-gated chloride channel (GluCl) selectively engineered to respond to the antihelmintic drug ivermectin. Additionally, we are further engineering the GluCl channel subunits to modify its ion selectivity from anionic to cationic. The resulting 'GluNa' channel enables us to stimulate neuronal activity. We will generate Adeno-associated virus driving expression of the subunits of both channels (GluNa and GluCl). We will inject these into the subthalamic nucleus or substantia nigra pars reticulata of both toxin-based and genetic mouse models of PD. We will study improvement of motor deficits in these mice by activation of the GluCl and GluNa channels by ivermectin. This will give insight whether DBS drives inhibition or activation of neurons and allow us to develop this further as an alternative therapy for late stage PD.

290. hERG K^+ channel drug block underlying acquired long-QT syndrome probed with natural and unnatural amino acid mutagenesis in mammalian cells

Fraser J. Moss, Shawna Frazier, Mark W. Nowak[1], Elisha Mackey[1], Nima Shiva[1], Dennis A. Dougherty[2]

Block of the human *ether-a-go-go* related gene (hERG) potassium channel is a safety concern for pharmaceutical development. By systematically mutating critical residues in the hERG pore to other natural and/or unnatural amino acids (UAAs), we aim to generate data that guide rational attempts to modify molecules to reduce hERG binding. Our studies are performed in mammalian cell lines, because a major limitation of the standard *Xenopus* oocyte model is the accumulation of test substances in the oocyte yolk, resulting in significant variability and discrepancies in potency estimates. To validate the methodology, we investigated the effect of the known hERG blockers risperidone, amperozide and sparfloxacin on the conventional hERG mutants T623S, S624A, S624T and Y652F when expressed in CHO cells. For each mutant, the shifts in IC_{50} for each drug compared to wild-type hERG were used to calculate $\Delta\Delta G$, the difference in the Gibbs free energy of association between the (drug and wild-type channel) and (drug and mutant channel). The $\Delta\Delta G$'s indicate the type of interaction between the drug and channel pore at each position, and for risperidone and amperozide were qualitatively similar to data previously generated in *Xenopus* oocytes. The next stage of our studies is to incorporate UAAs into hERG. We have identified a tyrosine aminoacyl-tRNA synthetase (bTyrRS)/tRNA ($bTyrtRNA_{CUA}$) pair from bacteria that rescue hERG expression when co-transfected with a hERG Y652TAG mutant in CHO cells. In control experiments, if either the tRNA synthetase or the tRNA plasmids are omitted from the transfection, no hERG expression is detected. The voltage dependence and waveforms of currents rescued by the bTyrRS/$bTyrtRNA_{CUA}$ pair were not significantly different from WT hERG. Directed evolution of the binding site of bTyrRS has resulted in the generation of a mutant that specifically aminoacylates our $bTyrtRNA_{CUA}$ with the UAA, cyclohexylalanine (CHA). The specific incorporation of CHA in Y652TAG and F656TAG hERG mutants expressed in CHO cells is presently under investigation.

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291. Fluorescent mGAT1 constructs reveal motifs important for correct trafficking and oligomerization

Fraser J. Moss, Princess. I. Imoukhuede, Jia Hu[*], Joanna L. Jankowsky, Michael W. Quick[*]

The protein-protein interactions, trafficking, and oligomerization of mGAT1, the mouse γ-aminobutyric acid (GABA) transporter, were studied using fourteen different fusions of mGAT1 with cyan, yellow or green fluorescent protein (CFP, YFP or GFP; collectively XFP). The constructs were expressed in neuroblastoma 2a cells, their function tested in a linear [^{3}H]GABA uptake assay, and their oligomerization probed with CFP-YFP Förster resonance energy transfer (FRET). To function like wild-type mGAT1, fusions of XFP to the mGAT1 C-terminus (mGAT1XFP) required their final three residues to constitute a naturally occurring PDZ-type II interacting motif. These wild-type-like mGAT1XFP fusions yielded up to 21% FRET efficiency, indicating efficient mGAT1 oligomerization. Furthermore, 44% FRET between one of these constructs and YFP-syntaxin-1A, which associates with the GAT1 N-terminus, indicated that the N- and C-terminals of mGAT1 oligomers are closer together than are adjacent C-terminals. Adding 45 hGAT1 C-terminal residues after mGAT1XFP resulted in little function, poor trafficking and no detectable FRET. An N-terminal XFP-mGAT1 fusion exhibited similar properties, indicating neither construct design oligomerized or trafficked to the plasma membrane. Inserting XFP between R565 and L566, resulted in 33% FRET but impaired function, indicating this region in the proximal C-terminus governs export from the endoplasmic reticulum but not oligomerization.

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292. Gamma-aminobutyric acid transporter GAT1 interacts with actin filaments via a C-terminal PDZ domain

Princess I. Imoukhuede, Fraser J. Moss, Darren J. Michael[*], Robert H. Chow[*]

Quantifying transporter trafficking is fundamental to understanding how released neurotransmitter is efficiently cleared from the synapse, through the modulation of local transporter density. Trafficking by lateral diffusion has been shown to significantly control post-synaptic receptor density. In this study, we determine how lateral mobility, the cytoskeleton, and the GAT1 C-terminus regulate transporter exchange on the plasma membrane. We apply fluorescence recovery after photobleaching (FRAP) on a γ-aminobutyric acid transporter-yellow fluorescent protein fusion (GAT1-YFP) transfected into N2a cells. Actin depolymerization increases the mobility of GAT1 by 10-30% and increases the time constant of fluorescence recovery by up to 60% compared to the non-treated control. Microtubule depolymerization, does not affect the lateral mobility of GAT1. Furthermore, the number of transporters freely diffusing in the membrane increases significantly when the PDZ-interacting domain is blocked. Thus, our data not only reveal a novel interaction between GAT1 and actin, but also suggest that this interaction is mediated through the GAT1-PDZ interaction.

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293. Rodent Aβ modulates the solubility and distribution of amyloid deposits in transgenic mice

Joanna L. Jankowsky, Linda H. Younkin[1], Victoria Gonzales[2], Daniel J. Fadale[1], Hilda H. Slunt[3], Steven G. Younkin[1], David R. Borchelt[3]

The amino acid sequence of APP is highly conserved, and age-related Aβ aggregates have been described in a variety of vertebrate animals with the notable exception of mice and rats. Three amino acid substitutions distinguish mouse and human Aβ that might contribute to their differing properties *in vivo*. To examine the amyloidogenic potential of mouse Aβ, we studied several lines of transgenic mice overexpressing wild-type mouse amyloid precursor protein (moAPP) either alone or in conjunction with mutant PS1 (PS1dE9). Neither overexpression of moAPP alone nor co-expression with PS1dE9 caused mice to develop Alzheimer-type amyloid pathology by 24 months of age. We further tested whether mouse Aβ could accelerate the deposition of human Aβ by crossing the moAPP transgenic mice to a bigenic line expressing human APPswe with PS1dE9. The triple transgenic animals (moAPP x APPswe/PS1dE9) produced 20% more Aβ but formed amyloid deposits no faster, and to no greater extent, than APPswe/PS1dE9 siblings. Instead, the additional mouse Aβ increased the solubility of accumulated amyloid in detergent and exacerbated amyloid deposition in the vasculature. These findings suggest that although mouse Aβ does not influence the rate of amyloid formation, the incorporation of Aβ peptides with differing sequences alters the solubility and localization of the resulting aggregates.

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294. Chronic nicotine cell specifically up-regulates functional α4* nicotinic receptors: Basis for both tolerance in midbrain and enhanced LTP in perforant path

Raad Nashmi, Cheng Xiao, Purnima Deshpande, Sheri McKinney[1], Sharon R. Grady[2], Paul Whiteaker[2], Qi Huang, Tristan McClure-Begley[2], Jon M. Lindstrom[1], Cesar Labarca, Allan C. Collins[2], Michael J. Marks[2]

Understanding effects of chronic nicotine requires identifying the neurons and synapses whose responses to nicotine itself, and to endogenous acetylcholine, are altered by continued exposure to the drug. In order to address this problem, we developed mice whose α4 nicotinic receptor subunits are replaced by normally functioning fluorescently-tagged subunits, providing quantitative studies of receptor regulation at micrometer resolution. Chronic nicotine increased α4 fluorescence in several regions; among these, midbrain and hippocampus were assessed functionally. Although the midbrain dopaminergic system dominates reward pathways, chronic nicotine does not change α4* receptor levels in dopaminergic neurons of VTA or substantia nigra pars compacta. Instead, up-regulated, functional α4* receptors localize to the GABAergic neurons of the VTA and substantia nigra pars reticulata. In consequence, GABAergic neurons from chronically nicotine-treated mice have a higher basal firing rate and respond more strongly to nicotine; because of the resulting increased inhibition, dopaminergic neurons have lower basal firing and decreased response to nicotine. In hippocampus, chronic exposure to nicotine also increases α4* fluorescence on glutamatergic axons of the medial perforant path. In hippocampal slices from chronically treated animals, acute exposure to nicotine during tetanic stimuli enhances induction of long-term potentiation in the medial perforant path, showing that the up-regulated α4* receptors in this pathway are also functional. The pattern of cell specific up-regulation of functional α4* receptors, therefore, provides a possible explanation for two effects of chronic nicotine: sensitization of synaptic transmission in forebrain and tolerance of dopamine release in midbrain.

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Publications

Ahern, C.A., Eastwood, A.L., Lester, H.A., Dougherty, D. and Horn, R. (2006) A cation-π interaction between extracellular TEA and an aromatic residue in potassium channels. *J. Gen. Physiol*. **128**:635-636.

Cashin, A.L., Torrice, M.M., McMeninen, K.A., Lester, H.A. and Dougherty, D. (2007) Chemical-scale studies on the role of a conserved aspartate in preorganizing the agonist binding site of the nicotinic acetylcholine reception. *Biochem.* **46**:630-639.

Damaj, M.I., Fonck, C., Marks, M.J., Deshpande, P., Labarca, C., Lester, H.A., Collins, A.C. and Martin, B.R. (2007) Genetic approaches identify differential roles for α4β2* nicotinic receptors in acute models of antinociception in mice. *J. Pharmacol. Exp. Ther*. **321**:1161-1169.

Drenan, R., Nashmi R., Imoukhuede, P., Just, H., McKinney, S. and Lester, H.A. (2007) Subcellular trafficking, pentameric assembly and subunit stoichiometry of neuronal nicotinic ACh receptors containing fluorescently-labeled α6 and β3 subunits. Submitted.

Imoukhuede, P.I., Moss, F.J., Michael, D.J., Chow, R.H. and Lester, H.A. (2007) Gamma-aminobutyric acid transporter GAT1 interacts with actin filaments via a C-terminal PDZ domain. Submitted.

Jankowsky, J.L., Younkin, L.H., Gonzales, V., Fadale, D.J., Slunt, H.H., Lester, H.A., Younking S.G. and Borchelt, D. (2007) Rodent Aβ modulates the solubility and distribution of amyloid deposits in transgenic mice. *J. Bio. Chem*. **282**:22707-22720.

Lerchner, W., Xiao, C., Nashmi, R., Slimko, E., van Trigt, L., Lester, H.A. and Anderson, D. (2007) Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated Cl^- channel. *Neuron* **54**:35-49.

Miwa, J.M., Stevens, T.R., King, S.L., Caldarone, B.J., Ibanez-Tallon, I., Cheng, X., Fitzsimonds, R.M., Pavildes, C., Lester, H.A., Picciotto, M.R. and Heintz, N. (2006) The prototoxin lynx 1 acts on nicotinic acetylcholine receptors to balance neuronal activity and survival *in vivo*. *Neuron* **51**(5):587-600.

Moss, F.J., Imoukhuede, P.I., Hu, J., Jankowsky, J.L., Quick, M.W. and Lester, H.A. Fluorescent mGAT1 constructs reveal motifs important for correct trafficking and oligomerization. Submitted.

Nashmi, R. and Lester, H.A. (2007) Cell autonomy, receptor autonomy, and thermodynamics in nicotine receptor upregulation. *Biochem. Pharmacol*. **74**:1145-1154.

Nashmi, R., Xiao, C., Deshpande, P., McKinney, S., Grady, S., Whiteaker, P., Huang, Q., McClure-Begley, T.D., Lindstrom, J., Labarca, C., Collins, A., Marks, M. and Lester, H.A. (2007) Cell specifically upregulated α4* nicotinic receptors: basis for both tolerance and cognitive sensitization. *J. Neurosci.* **27**:8202-8218.

Padgett, C.L., Hanek, A.P., Lester, H.A., Dougherty, D.A. and Lummis, S.C.R. (2007) Unnatural amino acid mutagenesis of the GABAA receptor binding site residues reveals a novel cation-π interaction between GABA and β_2Tyr97. *J. Neurosci*. **27**:886-892.

Pless, S.A., Dibas, M.I., Lester, H.A. and Lynch, J.W. (2007) Conformational variability of the glycine receptor M2 domain in response to activation by different agonists. *J. Biol. Chem*. In press.

Rodriguez, E.A., Lester, H.A. and Dougherty, D.A. (2007) Improved amber and opal suppressor tRNAs for incorporation of unnatural amino acids *in vivo*. Part 1: Minimizing misacylation. *RNA* **13**:1703-1714.

Rodriguez, E.A., Lester, H.A. and Dougherty, D.A. (2007) Improved amber and opal suppressor tRNAs for incorporation of unnatural amino acids *in vivo*. Part 2: Evaluating suppression efficiency. *RNA* **13**:1715-1722.

Shao, X.M., Wenbin, T., Xiu, J., Puskar, N., Fonck, C., Lester, H.A. and Feldman, J.L. (2007) α4* nicotinic receptors in preBötzinger complex mediate cholinergic/nicotinic modulation of respiratory rhythm. Submitted.

Tapper, A.R., McKinney, S.L., Marks, M.J. and Lester, H.A. Nicotine responses in hypersensitive and knockout α4 mice account for tolerance to both hypothermia and locomotor suppression in wild-type mice. *Physiol. Genom.*, August, (2007) Epub ahead of print.

Tapper, A.R., Nashmi, R. and Lester, H.A (2006) Neuronal nicotinic acetylcholine receptors and nicotine dependence in cell biology of addiction, J.D. Pollock, ed., (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press) **10**:179-191.

Teper, Y., Whyte, D., Cahir, E., Lester, H.A., Grady, S.R. Marks, M.J., Cohen, B.N., Fonck, C., McClure-Begley, T., McIntosh, J.M., Labarca, C., Lawrence, A., Chen, F., Gantois, I., Davies, P.J., Petrou, S., Murphy, M., Waddington, J., Horne, M.K., Berkovic, S.F. and Drago, J. (2007) Nicotine-induced dystonic arousal complex in a mouse line harboring a human autosomal-dominant nocturnal frontal lobe epilepsy mutation. *J. Neurosci*. **27**:10128-10142.

Wang, J., Lester, H.A. and Dougherty, D.A. (2007) Establishing an ion pair interaction in the homomeric ρ $GABA_a$ receptor that contributes to the gating pathway. *J. Biol. Chem*. **282**:26210-26216.

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Summary: Much of the research in this laboratory involves the study of interactions between the nervous and immune systems. Using knockout (KO) mice and over-expression *in vivo* with viral vectors, we are exploring the role of the neuropoietic cytokine leukemia inhibitor factor (LIF) in mouse models of Alzheimer's disease (AD) and multiple sclerosis (MS). This cytokine is being used to manipulate neural stem cell proliferation and fate in the adult brain. Also in the context of neuroimmune interactions, we are investigating a mouse model of mental illness based on the known risk factor of maternal influenza infection. Huntington's disease (HD) is another focus, where we are investigating potential therapies using intracellular expression of antibodies, and also manipulating NFκB activity. An additional project involves the inhibition of melanoma and glioma cell growth by endothelin receptor blockers.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group contributed to the discovery of a family that we have termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We have demonstrated that one of these cytokines, LIF, can coordinate the neuronal, glial and immune reactions to injury. Using both delivery of LIF *in vivo* and examination of the consequences of knocking out the LIF gene in mice, we find that this cytokine has a powerful regulatory effect on the inflammatory cascade. Moreover, LIF can regulate neurogenesis and gliogenesis. LIF is a critical regulator of astrocyte and microglial activation following stroke, seizure or trauma, and this cytokine also regulates inflammatory cell infiltration, neuronal and oligodendrocyte death, gene expression, as well as adult neural stem cell renewal. These results highlight LIF as an important therapeutic target. We are also examining the role of LIF in a transgenic mouse model of AD, where its administration can decrease the level of senile plaques, and in a chemical model of MS, where LIF can increase oligodendrocyte numbers.

Cytokine involvement in a new model for mental illness is also being investigated. This mouse model is based on findings that maternal infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, neuropathological, molecular and brain imaging methods to investigate the effects of activating the maternal immune system on fetal brain development and how this leads to altered behavior in young and adult offspring.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin (Htt), the protein that causes HD. We have produced single chain antibodies (scFvs) that bind to various domains of Htt, and these can either exacerbate or alleviate Htt toxicity in cultured cells, acute brain slices, and in a *Drosophila* HD model. Work has begun on viral vectors for delivering scFvs in a mouse model of HD. We have also implicated the NFκB signaling pathway in the pathogenesis of HD, and identified several steps in this signaling cascade as potential therapeutic targets.

295. Interleukin-6 mediates many of the effects of maternal immune activation on fetal brain development

Stephen Smith, Paul Patterson

Maternal infection by several different organisms has been implicated in the pathogenesis of schizophrenia. Maternal influenza infection or maternal immune activation (MIA) with the double-stranded RNA, poly(I:C), or with bacterial lipopolysaccharide (LPS) in rodents causes behavioral, histological and transcriptional changes in adult offspring. This indicates that MIA, rather than a specific pathogen, is responsible for the increased risk of mental illness in the offspring of mothers with infections during pregnancy. In investigating the possibility that cytokines may mediate the effects of MIA, we find that the cytokine interleukin-6 (IL-6) is essential for the manifestation of a variety of abnormalities in the adult offspring of poly(I:C)-treated mothers. Pregnant mice given a single injection of IL-6 on E12.5 show deficits in pre-pulse inhibition of the acoustic startle response (PPI) as well as deficits in latent inhibition (LI). Pregnant mice given an injection of poly(I:C) on E12.5 also show PPI, LI, exploratory and social interaction deficits, as previously reported. Co-administration of an anti-IL-6 neutralizing antibody with poly(I:C) prevents all of these deficits, while co-administration of anti-interferon-γ or anti-IL-1β does not. Anti-IL-6 also prevents maternal poly(I:C)-induced changes in gene expression in the adult frontal cortex. Finally, maternal

injection of poly(I:C) in IL-6 knockout mice does *not* cause behavioral deficits in the offspring. Thus, IL-6 is necessary for MIA to produce autism- and schizophrenia-like behaviors that are manifested in the adult offspring. Current research is exploring the site(s) of IL-6 action.

296. Information processing in the hippocampus of the offspring of immune-activated mothers

Hiroshi Ito, Stephen Smith

The offspring of mice whose immune systems were activated by injection of the dsRNA, poly(I:C), show behavioral abnormalities. Several of these behaviors, including increased responses to low doses of amphetamine, as well as disrupted latent inhibition, suggest altered function of hippocampal and dopamine systems. To examine this possibility, we made hippocampal slices from adult mice born to control or immune-activated mothers, and measured the electrophysiological responses of CA1 pyramidal neurons. The offspring of poly(I:C)-treated mice show increased amplitude and decreased frequency of spontaneous miniature excitatory post-synaptic currents, suggesting abnormal synaptic structure or function. Dopamine sensitivity in CA1 is of interest because dopamine is known to depress excitatory responses at temporoammonic-CA1 synapses. Compared to controls, we find that dopamine-induced excitatory depression is significantly larger in the slices from offspring of poly(I:C)-treated mothers, suggesting higher sensitivity to dopamine. Taken together, our physiological data suggest that the offspring of poly(I:C)-treated mothers exhibit abnormal information processing in the hippocampus.

297. Maternal immune activation impairs extinction of the conditioned eyeblink response in the adult offspring

Ka Hung Lee[*], Stephen Smith, Soyun Kim[1], Paul H. Patterson, Richard F. Thompson[1]

We are investigating classical eyeblink conditioning, a behavior that is abnormal in autistic subjects, in the adult offspring of poly(I:C)-injected mice. Pregnant mice are injected with polyI:C or saline on embryonic day 12.5, and adult offspring are tested in a delay eyeblink conditioning paradigm. Compared to the saline group, the offspring of the poly(I:C)-activated mothers show impaired extinction of the conditioned response. Auditory brainstem responses and tail-flick latency are similar in both groups, indicating normal auditory and somatosensory functions. The impaired extinction in the poly(I:C) group suggests that perseverative motor behavior, a characteristic of autism, is induced in the offspring by maternal immune activation.

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298. Maternal influenza infection alters fetal brain development

Limin Shi, Natalia Malkova, Yixuan Andy Su[*], Paul H. Patterson

Epidemiological studies have shown that maternal infection can increase the risk for mental illness in the offspring. In a mouse model of maternal respiratory infection with influenza virus, the adult offspring display striking behavioral, pharmacological and histological abnormalities. In addition to a localized loss of Purkinje cells that is strikingly similar to a common pathology in autism, we find an altered migration of neurons in the fetal cortex. Whereas late-born cortical neurons are appropriately positioned in the upper layers in sham control offspring and naïve mice from postnatal day 0 to young adulthood, the late-born cortical neurons in the offspring of infected mothers are abnormally distributed. The degree of this effect depends on viral dose, maternal sickness behavior and inoculation methods. This neuropathology is of particular interest because it is very similar to that reported for embryos whose level of disrupted in schizophrenia-1 (DISC1) mRNA is knocked down. It is possible that our finding in the mouse model is relevant to the pathology and behavioral abnormalities that have been linked to the cerebral cortex in schizophrenia.

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299. The effect of prenatal influenza infection on neurobehavioral development of mouse offspring

Natalia Malkova, Paul H. Patterson

We are investigating the neurobehavioral development of mouse pups born to mothers whose immune systems were activated at mid-gestation. Tests include the rate of ultrasonic vocalizations (which is important for mother-infant social interaction), maturation status of neuromotor reflexes, and neurogenesis. We find that injection of double-stranded RNA (poly(I:C)), which evokes an inflammatory response in the mother similar to that induced by influenza virus, alters the behavior of the offspring. Compared to controls, 10 day-old C57BL/6J pups born to mothers given poly(I:C) (on E12.5) have lower rates of ultrasound calling when separated from their mothers. Analysis of temporal organization of pup ultrasonic vocalizations shows that pups born to poly(I:C)-treated mothers emit more single calls than calls in bouts compared to control ones. The latency period in the isolation test does not differ in these groups. The absence of detectable differences in maternal responsiveness towards the pups suggests that the fewer distress calls emitted by pups are the result of a reduced sensitivity to isolation. It is known that short maternal deprivation in 10 day-old rat pups leads to dopamine release in the septum and striatum (Kehoe *et al*., 1996). Therefore, studies are in progress to study the dopaminergic system of pups born to mothers whose immune systems were activated at mid-gestation.

300. Novel intrabodies block aggregation and toxicity of mutant huntingtin by increasing its turnover

Amber L. Southwell, Ali Khoshnan, Paul H. Patterson

Huntington's disease (HD) is a neurodegenerative disorder caused by the expansion of a polyglutamine (polyQ) tract in the huntingtin protein (Htt). This expansion leads to Htt aggregation and degeneration of medium sized spiny neurons of the striatum. We are investigating the use of intrabodies (intracellularly expressed antibodies) as potential therapeutics for HD. Intrabodies have great therapeutic potential due to their specificity in target recognition, and are currently being developed for the treatment of HIV and cancer. Single chains (scFvs) consisting of the variable heavy and light antigen recognition domains (V_H and V_L) connected by a linker, or single domains consisting of either V_H or V_L alone are preferable for this type of application due to their small size and lack of antigenicity. Intrabodies that recognize the expanded polyglutamine tract increase mutant Htt (mHtt)-induced aggregation and cell toxicity in culture. Conversely, intrabodies that recognize regions that flank the poly-Q tract, including the 17 N-terminal amino acids (V_L12.3) or the polyproline (polyP) domains (MW7), inhibit mHtt aggregation and promote cell survival in culture. Moreover, MW7 inhibits mHtt-induced striatal neuron death in acute brain slices and in *Drosophila* HD models. However, MW7 requires a 4:1 ratio to mHtt for optimal effects. The therapeutic benefits of MW7 derive from binding to the polyP domains of Htt, and these domains are important for mHtt toxic gain of function. In an effort to generate a more efficacious intrabody with the benefits of binding near the polyP region, we produced novel intrabodies (Happs) against the MPD (multiple proline domain; two polyP stretches separated by a P-rich domain) of Htt. Happ1 and 3 are V_Ls that bind to mHtt in an MPD-dependent manner. We compared the Happs with MW7 and V_L12.3 for efficacy in blocking mHtt exon 1 (mHDx-1) aggregation and toxicity, as well as their effects on mHDx-1 turnover and localization. The Happs are intermediate in efficacy in blocking aggregation and toxicity of mHDx-1, with V_L12.3 being the most effective. An attractive feature of the MPD-binding intrabodies is, however, their ability to reduce mHtt toxicity by increasing its turnover. This is in contrast to V_L12.3, which stabilizes soluble mutant HDx1 and enhances its nuclear localization. We propose that the MPD of mutant HDx1 regulates its stability, and that compromising this function with Happ binding represents a novel therapeutic strategy for treating HD.

301. Interaction of mutant huntingtin with the NF-κB pathway

Ali Khoshnan, Simona Tescu[*], Jan Ko

Transcriptional dysregulation by mutant huntingtin (Htt) protein has been implicated in the pathogenesis of Huntington's disease (HD) and we found that mutant Htt activates the NF-κB pathway. Mutant Htt physically associates with IKKγ, a regulatory component of the IκB kinase complex (IKK). In cultured cells, this interaction results in the activation of IKK, leading to the phosphorylation and degradation of the inhibitory protein, IκBα. These findings have *in vivo* relevance, as striatal extracts from HD transgenic mice have higher levels of IKK than extracts from control mice, and activated NF-κB is found in the nucleus of striatal and cortical neurons in HD mice. Binding to IKKγ is mediated by the expanded polyglutamine stretch in mutant Htt, and is augmented by the proline-rich motifs of Htt. Expression of IKKγ promotes mutant Htt aggregation and nuclear localization. Conversely, an N-terminally truncated form of IKKγ, which interferes with IKK activity, blocks Htt-induced NF-κB activation and reduces the toxicity of mutant Htt in cell culture and in an acute brain slice model of HD. Toxicity is also inhibited by expression of a mutant F-box deleted E-3 ubiquitin ligase, ΔF-βTRCP, which specifically blocks degradation of IκB inhibitory proteins. Thus, aberrant interaction of mutant Htt with IKKγ, and subsequent NF-κB activation, may be important for HD pathology. IKKγ also regulates expression of non-NF-κB target genes. In neurons, a free pool of IKKγ is present in the nucleus. Using cDNA microarrays, we find that expression of IKKγ from a lentivirus influences the levels of many genes implicated in neuronal survival and differentiation. One of these targets is the neurotrophic factor, pleiotrophin (PTN). The level of PTN is suppressed by IKKγ, but induced in cells expressing mutant Htt. Over-expression of PTN reduces aggregation and toxicity of mutant Htt in a neuronal model of HD. Studies are in progress to examine the signaling pathways that contribute to PTN-induced inhibition of mutant Htt toxicity.

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302. The role of IκB-kinase complex in neuronal development and function

Ali Khoshnan, Jan Ko

The role of IκB-kinase complex (IKK) in neuronal development, survival and degeneration is not understood. In non-neuronal cells IKK regulates the activity of the transcription factor NF-κB. The core components of the IKK complex include two serine-threonine kinases IKKα (IKK1) and IKKβ (IKK2), and a regulatory non-catalytic module, IKKγ (NEMO). IKKα and IKKγ also have NF-κB independent functions. We showed that binding of mutant Htt to IKKγ leads to aberrant IKK activity and enhances mutant Htt neurotoxicity (Khoshnan *et al.*, 2004). On the other hand,

mice deleted for both IKKα and IKKβ die at E12 and display enhanced apoptosis in the neuroepithelium, indicating normal and regulated activity of IKK complex is essential for development and function of neurons. We are investigating the function of IKKs in neuronal function and development. We find that IKKα promotes neurite outgrowth in differentiating rat cortical stem cells and it is found in the growing tips of neurites. Growth cone cues such as netrin activate IKK. IKKα is activated in response to neuroprotective molecules such as IGF and estrogen, suggesting a role in neuronal survival. IGF-1 promotes nuclear localization of IKKα, where it colocalizes with CREB binding protein. Human neurons have constitutive IKKα activity and treatment with DNA damaging agents reduces both its level and activity. Reduction of IKKα is mediated at the mRNA level and is dependent on the activity of p53 tumor suppressor protein. Neurons engineered to express IKKα from a lentivirus are more resistant to DNA damage-induced cell death. IKKα induces expression of HSP70 and BDNF, and protects against DNA damage-induced cleavage of huntingtin (Htt) protein. Studies are in progress to dissect the signaling pathways that are influenced by IKKα in neurons and how they may induce neuroprotection. To explore the role of IKKβ in the CNS, we are deleting IKKβ in the mouse brain by Cre-lox technology. These mice will be tested for cytokine-mediated behavioral abnormalities as well as Htt-induced neurodegeneration.

303. Direct selection of neuroprotective intrabodies for Huntington′s disease

Charles W. Bugg, Paul H. Patterson

Death of neurons in the striatum and cortex is a key component of Huntington′s disease (HD), a progressive and fatal neurodegenerative disorder. Several intracellular antibodies (intrabodies) have been developed as potential therapeutics for HD, based on their binding to mutant huntingtin (mHtt) protein. These intrabodies ameliorate the disease phenotype with various efficiencies in cell culture. Some even exacerbate the cell death. We are developing a more direct, iterative system to select for intrabodies that prevent mHtt-induced cell death without necessarily binding mHtt. A library of intrabodies is expressed in PC12 cells carrying mHtt exon 1 (HDx1) with an expanded polyglutamine repeat (103Q) under control of the ecdysone promoter. After induction of the toxic HDx1, only cells carrying neuroprotective intrabodies will survive, and those intrabody sequences can be recovered, amplified and recycled through the selection. The efficiency of the selection will be determined in large part by the ability to kill nearly all cells that do not carry a neuroprotective sequence. After optimization of conditions, we find that 99% of the PC12/PQ103 cells die within 12 days of induction with 5μM Muristerone A. Two library strategies are being pursued. A mutagenized intrabody pool will be used to directly transfect the PQ103-inducible PC12 cells. In parallel, the intrabody library will be cloned into the lentiviral vector pFUGW and used to produce a lentiviral library with which the PC12 cells will be transduced. As proof of principle, we will select a published intrabody that functions in the absence of the conserved disulfide bond. This system will then be used to select novel intrabodies that protect from mHtt-induced cell death for use as therapeutics for HD.

304. Adenoviral delivery of leukemia inhibitory factor stimulates oligodendrocyte progenitor cell proliferation in the context of CNS demyelination

Benjamin E. Deverman, Sylvian Bauer

Multiple sclerosis (MS) is characterized by motor, sensory and cognitive deficits that result from focal demyelinated lesions in the brain and spinal cord. These lesions are often remyelinated in early stages of relapsing-remitting MS by newly generated oligodendrocytes derived from oligodendrocyte progenitor cells (OPCs). While remyelination can be efficient, it is often inconsistent and is largely absent in the chronic lesions that accumulate during the course of this progressive disease. Currently, there is a paucity of therapies aimed at promoting remyelination. The development of such therapies is crucial since available treatments aimed at slowing the course of demyelination are largely ineffective in the progressive stage of the disease. Factors that stimulate OPC proliferation and/or encourage oligodendrocyte maturation are being investigated for their potential use as treatments aimed at promoting remyelination and ameliorating clinical deficits. One potential factor whose role in remyelination remains unexplored is leukemia inhibitory factor (LIF). We find that injection of an adenovirus expressing LIF into the lateral ventricle of adult mice strongly stimulates the proliferation of OPCs in the corpus callosum, cortex, striatum and septum. Based on this finding, we are investigating whether exogenous LIF, delivered either alone or together with additional factors, will stimulate OPC proliferation and in turn enhance remyelination in the context of a chronic cuprizone demyelination model. Our preliminary results suggest that LIF induces the proliferation and accumulation of OPCs in regions of extensive demyelination, including the hippocampus and medial corpus callosum. We are now analyzing later time points to determine whether these newly generated OPCs differentiate into mature myelinating oligodendrocytes.

305. Neurotoxicity of manganese oxide nanomaterials

Diana Stefanescu[*]*, Ali Khoshnan, Janet Hering*[*]

Chronic exposure to manganese-containing dust particles induces neurological, respiratory and reproductive abnormalities. Manganese accumulation in certain brain regions following elevated exposure can lead to a disorder termed manganism. The reactivity of manganese oxide-containing nanoparticles with biological systems may regulate their neurotoxicity. To better understand their mechanism of action, we are exploring the effects of manganese oxide nanomaterials on a rat

neuronal cell line. Transmission electron microscopy reveals that after 48 hr, nanomaterials penetrate into the cytoplasm of the cells, independently of the size of the material or its aggregation state. Our initial data suggest that exposure to Mn_3O_4 causes dose-dependent cell death, which is influenced by particle size and time of exposure. As expected, smaller nanoparticles show higher toxicity than larger ones. The toxic effect is greater on proliferating cells than on non-dividing, differentiated neurons. Upon treatment with nanoparticles, reactive oxygen species (ROS) are formed, activating the transcription factor NF-κB. Both ROS formation and NF-κB activation are particle concentration- and composition-dependent. However, NF-κB inhibitors do not block the Mn_3O_4-induced cell death. Studies are in progress to examine the mechanism of Mn_3O_4-induced cell death.

Environmental Science and Engineering, Caltech

306. The effect of endothelin receptor antagonists on cancer cells

Jennifer Montgomery

We are investigating the effect of two distinct endothelin receptor B (ETRB) antagonists on the proliferation of melanoma and glioma cells. Previous work in this laboratory indicated that the ETRB antagonist BQ788 decreases melanoma cell proliferation *in vitro* and *in vivo*. We have extended this study to a number of glioma cell lines and find that two ETRB-specific antagonists, A-192621 and BQ788, reduce the number of viable cells in a dose- and time-dependent manner. The more potent of the two antagonists, A-192621, decreases the mean number of cell divisions at least in part by inducing a G2/M arrest and apoptosis. Microarray analysis of the effects of A-192621 treatment reveals up-regulation of several DNA damage-inducible genes. These results were confirmed by real-time RT-PCR. Importantly, reducing expression of ETRB with small interfering RNAs does not abrogate the effects of either A-192621 or BQ788 in glioma or melanoma cells. Furthermore, BQ123, an endothelin receptor type A-specific antagonist, has no effect on cell viability in any of these cell lines, indicating that the ETRB-independent effects on cell viability exhibited by A-192621 and BQ788 are not a result of ETRA inhibition. Thus, while ETRB antagonists reduce the viability of glioma cells *in vitro*, it appears unlikely that this effect is mediated by ETRB inhibition or cross-reaction with ETRA.

Publications

Bauer, S. and Patterson, P.H. (2006) Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain. *J. Neurosci.* **26**:12089-12099.

Bauer, S. Kerr, B. and Patterson, P.H. (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. *Nature Rev. Neurosci.* **8**:221-232.

Greger, B., Kateb, B., Gruen, P. and Patterson, PH (2007) A chronically implantable, hybrid cannula-electrode device for assessing the effects of molecules on electrophysiological signals in freely behaving animals. *J. Neurosci. Meth*. **163**:321-325.

Holmberg, K.H. and Patterson, P.H. (2006) Leukemia inhibitory factor is a key regulator of astrocytic, microglial and neuronal responses to seizure. *Brain Res*. **1075**:26-35.

Montgomery, J.P. and Patterson, P.H. (2006) Behavioral stress and tumor progression. *Anticancer Res*. **26**:1189-1192.

Patterson, P.H. (2006) Modeling features of autism in animals. In: *Understanding Autism: From Basic Neuroscience to Treatment*, Moldin, S.O. and Rubenstein, J.L.R., (Eds.), Taylor & Francis, Boca Raton, FL, pp. 277-302.

Patterson, P.H. (2006) Pregnancy, immunity, schizophrenia and autism. *Eng. & Sci.* **69**:10-21.

Patterson, P.H., Xu, W. and Deverman, B.E. (2007) Maternal immune activation, cytokines and autism. In: *Autism: Current Theories and Evidence*, Zimmerman, A.W. (Ed.), Humana Press, Totowa, NJ. In press.

Smith, S.E.P., Li, J., Garbett, K., Mirnics, K. and Patterson P.H. (2007) Maternal immune activation alters fetal brain development through interleukin-6. *J. Neurosci*. In press.

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Summary: Synapses, the points of contact and communication between neurons, can vary in their size, strength and number. The ability of synapses to change throughout the lifetime of the animal contributes to the ability to learn and remember. We are interested in how synapses are modified at the cellular and molecular level. We are also interested in how neuronal circuits change when synapses change their properties. We conduct all of our studies in the hippocampus, a structure known to be important for memory in both humans and animals. We use molecular biology, electrophysiology and imaging to address the questions detailed below.

A major focus of the lab concerns the cell biological mechanisms that govern modifications at individual synaptic sites. In particular, we are interested in the idea that dendritic protein synthesis and degradation may contribute to synaptic plasticity. We are also interested in mRNA and protein trafficking during synaptic plasticity.

We are also examining the role of the cadherins family of cell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses in the hippocampus. Earlier, we demonstrated that function-blocking cadherin antibodies or peptides can prevent long-term potentiation, without interfering with basal synaptic transmission. We hypothesize that cadherin bonds may be sensitive to local fluxes in extracellular calcium imposed by action potential activity. We are now examining the molecular mechanisms by which cadherins influence synaptic strength and the involvement of cadherins in the formation and maintenance of synapses, using fluorescence resonance energy transfer and endocytosis assays.

A unique endeavor in the lab involves the recording of single neuron activities in the medial temporal lobe of human epilepsy patients. In these studies, we are able to correlate single neuron responses with behavioral experience and performance. These studies should elucidate some fundamental mechanisms of brain coding and representation.

307. eEF2 couples weak synaptic activation to suppression of dendritic translation

Michael A. Sutton, Anh Pham, Anne M. Taylor, Hiroshi T. Ito, Erin M. Schuman

Local protein synthesis in neuronal dendrites has emerged as a powerful mechanism for regulating both basal synaptic function and activity-dependent changes in synaptic efficacy. We have previously reported that ongoing weak synaptic activation conferred by miniature synaptic transmission (minis) inhibits local translation in dendrites of hippocampal neurons (Sutton *et al*., 2004) and that this regulation acts to stabilize synaptic function during activity blockade (Sutton *et al*., 2006). Here, we show that the coupling of minis to the translational machinery in dendrites is mediated, in part, by local regulation of eukaryotic elongation factor (eEF2). eEF2 catalyzes ribosomal translocation during polypeptide elongation, and its activity is strongly inhibited by phosphorylation. In the absence of action potential (AP)-evoked neurotransmission, mini blockade inhibits eEF2 phosphorylation, whereas acute stimulation of mini frequency enhances eEF2 phosphorylation. Consistent with a role for mini-induced eEF2 phosphorylation in mediating translational suppression, pharmacological disruption of eEF2 kinase stimulates dendritic synthesis of a fluorescent translation reporter, and restricted perfusion of eEF2 kinase inhibitors produces local translational activation in treated dendritic segments. These results thus indicate that eEF2 serves as a local biochemical sensor designed for coupling weak synaptic activation with local translational suppression in dendrites.

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308. Monitoring global and local fates of activity-induced proteomes using fluorescent non-canonical amino acid-tagging (FUNCAT)

Daniela C. Dieterich, Jennifer J. Lee, Anne M. Taylor, John Ngo[1], David A. Tirrell[2], Erin M. Schuman

Both global and local protein synthesis have been implicated in different forms of synaptic plasticity. However, the selective identification and visualization of a newly synthesized proteome has been hindered by the basic fact that all proteins, new and old, share the same pool of amino acids and thus, are chemically

indistinguishable. Recently, we developed bioorthogonal non-canonical amino acid tagging (BONCAT) for the specific identification of newly synthesized proteins. This approach is based upon the co-translational introduction of small bioorthogonal chemical groups via the incorporation of the non-canonical amino acids Azidohomoalanine (AHA) or Homopropargylglycine (HPG) into proteins and the chemoselective-tagging of labeled proteins with an affinity tag via [3+2] click chemistry. Here, we demonstrate the extension of this approach to visualize newly synthesized proteins using fluorescent tags (FUNCAT) in cultured hippocampal neurons.

Incorporation of modified amino acids is unbiased, non-toxic and does not increase protein degradation. While abundant signal is detected in neurons treated with the modified amino acids, no FUNCAT signal is detected in protein synthesis inhibitor-treated cultures, confirming that this procedure labels newly synthesized proteins with high specificity. Strikingly, newly synthesized proteins in the somata of cultured hippocampal neurons can be detected as early as 10 min after AHA-or HPG incubation; a steady increase in protein synthesis is observed over time. Signal in proximal dendrites can be detected after a 20 min incubation with AHA or HPG. To examine the effects of synaptic activation on protein synthesis, we conducted experiments using the neurotrophin BDNF. After bath application of BDNF (50 ng/ml) for 60 min, we are able to detect a 1.6-fold increase in the signal of newly synthesized proteins in proximal segments of BDNF-treated dendrites when compared to vehicle-treated controls. Moreover, we demonstrate that local application of AHA and HPG can be used to evaluate the extent and fate of locally synthesized proteins using micromanipulator-assisted local perfusion and microfluidic chambers. Finally, a pulse-chase-like application of AHA and HPG allows monitoring of proteins synthesized in two sequential time periods. In conclusion, FUNCAT makes it possible to address the temporal and spatial characteristics of newly synthesized proteomes under basal, as well as elevated, levels of synaptic activity.

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309. Identifying and visualizing the hippocampal dopaminergic subproteome using BONCAT and FUNCAT

Jennifer J. Lee, Daniela C. Dieterich, John Ngo[1], David A. Tirrell[2], Erin M. Schuman

Both synaptic and behavioral plasticity require new protein synthesis. Dopamine is a critical neuromodulator, and abnormalities in dopaminergic regulation underlie disorders like Parkinson's disease, Alzheimer's disease, and schizophrenia - diseases that impair the ability to form and retrieve memories. The stimulation of D1/D5 dopaminergic receptors in the hippocampus is thought to be critical for protein synthesis-dependent long-term potentiation (LTP), a process important for long-term synaptic plasticity and memory. Despite considerable effort, the proteins synthesized upon activation of dopaminergic pathways, the dopaminergic subproteome, are still largely unknown.

Using bioorthogonal noncanonical amino acid-tagging (BONCAT), we are able to specifically identify components of the hippocampal dopaminergic proteome in an unbiased, non-toxic manner. Moreover, we utilize a sister technique, fluorescent noncanonical amino acid tagging (FUNCAT), to visualize the effect of D1/D5 dopaminergic receptor stimulation in regulating global and local protein synthesis in the hippocampus. Both techniques employ a methionine surrogate, azidohomoalanine (AHA), which after translational incorporation into proteins is conjugated to an azide-bearing tag via [3+2] copper-catalyzed click chemistry. Upon stimulation with a D1/D5 dopamine receptor-specific agonist, there are significantly increased levels of FUNCAT signal present in dendrites when compared to unstimulated dendrites. Since our interests also extend to dendritic protein synthesis, we have combined the use of FUNCAT with a Transwell culture system and have observed protein synthesis in both the somatic and dendritic compartments. Therefore, we demonstrate the application of BONCAT and FUNCAT to probe one of the protein synthesis-dependent functions in the hippocampus.

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310. Investigating local processing in dendrites using microfluidic perfusion chambers

Anne M. Taylor, Daniela C. Dieterich, Erin M. Schuman

Synaptic plasticity involves local and coordinated changes in both the presynaptic and postsynaptic regions of the synapse. In particular, recent evidence shows that local protein synthesis in dendrites is required for some forms of synaptic plasticity. The underlying molecular and structural mechanisms of synaptic plasticity are the focus of extensive studies using a variety of different neuronal culture systems. Dissociated hippocampal neurons provide an important tool for investigating local changes in synaptic activity with particular relevance to learning and memory. The recent use of microfluidic devices provides an important improvement for the organization and manipulation of dissociated hippocampal neurons that extend processes long distances with random orientations. Here we develop and use a microfluidic device to investigate local processing in dendrites. Using this

microfluidic device we are able to spatially orient dendrites and axons in a highly structured/organized manner, while enabling both pharmacological and genetic manipulations. This microfluidic device consists of a series of microgrooves that align dendrites and axons in parallel orientation. We show that dendrites and axons form synaptic contacts in these microgrooves. The use of a perfusion channel running perpendicular to the microgrooves allows a stable and locally restricted pharmacological agent to a distinct dendritic region in these devices with a resolution of <30 μm. The concentration of the perfusion stream reaches equilibrium within 1 min and can be washed out within this same timeframe. A sequential series of molecules can be perfused numerous times. Further characterization work includes the diffusion of molecules through the dendrite during the perfusion. This work provides a significant technical improvement for investigating local processing at both pre- and postsynaptic regions.

311. Activity-dependent regulation of ribosomes in rat hippocampal neurons

Young J. Yoon, Erin M. Schuman

The requirement for new protein synthesis has long been associated with synaptic plasticity, as well as learning and memory. In addition, the presence of ribosomes in dendrites and axons strongly suggests that translation can occur locally to modulate nearby synapses in response to synaptic activity. To investigate whether ribosomes can translocate in and out of synaptic compartments, we have analyzed postsynaptic densities (PSD) prepared from acute hippocampal slices from rats. We observed that under basal conditions, numerous ribosomal proteins were associated with PSDs in the triton-resistant fraction, suggesting a specific interaction. Interestingly, treatment of slices with BDNF led to increased amounts of ribosomal proteins in PSDs, supporting the idea that ribosomes can translocate or redistribute to PSDs upon stimulation of translation by neurotrophins. In addition, we examined whether chronic blockade or stimulation alters dendritic localization of ribosomal proteins. We posited that a prolonged exposure of neurons to pharmacological agents could provide clues to global regulation of the translation machinery. Our data suggests that chronic blockade of action potentials by TTX reduced the presence of the small 40S subunit in dendrites, while the GABA receptor antagonist bicuculline led to an increase in the large 60S subunit levels. These results support the idea that hippocampal neurons can modulate ribosome levels in response to changes in activity.

312. Activity-dependent regulation of proteasome assembly and biogenesis in rat hippocampal neurons

Hwan-Ching Tai, Kuang-Jung Chang, Baris Bingol, Erin M. Schuman

Targeted degradation of proteins in eukaryotes is largely regulated by the ubiquitin-proteasome system (UPS). Many studies have demonstrated the importance of UPS-mediated protein degradation in synaptic plasticity, via both presynaptic and postsynaptic mechanisms. The UPS, a complex biochemical pathway, is regulated at multiple levels in the neuron. Previous studies have emphasized the role of ubiquitination and deubiquitination enzymes in neuronal function, but little is known about the regulation of proteasome itself. Our laboratory previously demonstrated that proteasome localization can be regulated by neuronal activity, rapidly translocating into dendritic spines after depolarization. This prompted us to investigate whether proteasome composition and levels also undergo activity-dependent changes. In rat hippocampal neurons, we observed that proteasome assembly and biogenesis are regulated by synaptic activity, which may contribute to activity-dependent restructuring of synapses.

313. Activity-regulated N-cadherin endocytosis

Chin-Yin Tai, Shreesh P. Mysore, Erin M. Schuman

Enduring forms of synaptic plasticity are thought to require ongoing regulation of the adhesion molecules present at synaptic junctions. One poorly understood aspect of synaptic adhesion is the activity-regulated trafficking of adhesion molecules, a process which is linked to structural remodeling. N-cadherin is a synaptic adhesion molecule known to be critical for the functional and structural integrity of synapses. Here we demonstrate that N-cadherin, present on the surface of neurons, undergoes a surprisingly high basal rate of internalization. However, upon activation of NMDA receptors (NMDAR), the rate of N-cadherin endocytosis is significantly reduced, resulting in an accumulation of N-cadherin at the plasma membrane. β-catenin, an N-cadherin binding partner, is a primary regulator of N-cadherin endocytosis. Following NMDAR stimulation, β-catenin accumulates in spines and exhibits increased binding to N-cadherin. When a mutant form of β-catenin (Y654F, exhibiting greater affinity to N-cadherin) is expressed in neurons, the NMDAR-dependent regulation of N-cadherin internalization is abolished, and the surface population N-cadherin is stabilized. Furthermore, prolonged stabilization of N-cadherin at the surface blocks NMDAR-dependent synaptic plasticity. These results indicate that NMDAR activity regulates N-cadherin endocytosis, providing a mechanistic link between structural plasticity and persistent changes in synaptic efficacy.

314. Spatiotemporal dynamics of cadherin adhesion at synapses and adherens junctions

Sally A. Kim, Chin-Yin Tai, Eric A. Mosser, Erin M. Schuman

Cadherins are a major family of calcium-dependent cell-cell adhesion molecules that play a role in the control of structural and synaptic plasticity. To directly test whether synaptic activity dynamically regulates the adhesive strength of cadherin interactions across the synapse, we have developed a Förster

Resonance Energy Transfer (FRET) reporter system for visualizing homophilic interactions of cadherin across adherens junctions. Towards this end, we created N-cadherin fusion proteins with an intramolecular fluorescent protein insertion (in two colors-cerulean and venus) using a transposon-mediated insertion method. By screening over 500 clones, we were able to generate a fluorescently-labeled N-cadherin that is functional as assessed by a calcium-dependent L-cell aggregation assay, exhibits fluorescence of sufficient intensity for FRET, mimics the cellular localization of endogenous N-cadherin, and interacts with the correct binding target, β-catenin. We can probe both *cis*- and *trans*-cadherin interactions in heterologous cells using FRET depending on whether the cells are co-transfected with both constructs or if neighboring cells are expressing one of each. To transfer our FRET reporter system into neurons, N-cadherin constructs are expressed using viral gene delivery such that a FRET donor (cerulean-cadherin) is expressed in presynaptic neurons while a FRET acceptor (venus-cadherin) is expressed in postsynaptic neurons. FRET will then be used to detect cadherin interactions between pre-and postsynaptic cells under various conditions. In particular, we will examine the effects of synaptic activity and varying extracellular calcium concentrations on cadherin-cadherin dynamics. These experiments will test our hypothesis that cadherins act as extracellular calcium detection system to coordinate synaptic plasticity across the synapse.

315. Frequency-dependent gating of signal transmission and synaptic plasticity by dopamine at temporoammonic-CA1 synapses

Hiroshi T. Ito, Erin M. Schuman

CA1 pyramidal neurons receive two distinct excitatory synaptic inputs, one is from area CA3 (Schaffer-collateral pathway) and the other is from the entorhinal cortex (temporoammonic pathway). The interaction between these inputs is likely of great importance for the hippocampal function. Here, in recordings from hippocampal slices, we show that DA acts specifically at the temporoammonic pathway to filter the excitatory drive onto pyramidal neurons based on the input frequency. During low frequency patterns of stimulation, DA depressed excitatory temporoammonic inputs to both CA1 pyramidal neurons and local inhibitory GABAergic interneurons via presynaptic inhibition. In contrast, during high frequency patterns of stimulation, DA potently facilitated the temporoammonic excitatory drive onto CA1 pyramidal neurons, owing to diminished feed-forward inhibition. Analysis of DA's effects over a broad range of stimulus frequencies indicates that it acts as a high-pass filter, augmenting the response to high-frequency inputs while diminishing the impact of low frequency inputs. Furthermore, DA-induced disinhibition has a profound influence on synaptic plasticity at both Schaffer-collateral-CA1 and temporoammonic-CA1 synapses. Taken together, our data demonstrate that DA acts as a gate on the direct cortical input to the hippocampus, modulating information flow and synaptic plasticity in a frequency-dependent manner.

316. Activity of human hippocampal-amygdala neurons during retrieval of episodic memories

Ueli Rutishauser, Adam N. Mamelak, Erin M. Schuman

Episodic memories allow us to remember not only that we have seen an item before (familiarity) but also where and when we have seen it (context). Sometimes, we are confident that an item is familiar but cannot recollect where or when it was seen. Thus, the two components of episodic recall, familiarity and recollection, can be behaviorally dissociated. It is well known that the medial temporal lobe (MTL) plays an important role in this process. It is not clear, however, whether these two components of memory are represented separately by distinct brain structures or different populations of single neurons in a single anatomical structure. We recorded single neurons in the hippocampus and amygdala of patients implanted with depth electrodes for the purpose of localizing epileptic seizure origins. Previously we identified a class of neurons that changed its firing rate to the second presentation of a previously novel stimulus (single-trial learning). Here we investigate the activity of the same subpopulation of neurons during episodic memory retrieval. We find that the spiking activity of single neurons in the human hippocampus and amygdala contain information about both components of memory. We found that the neuronal activity evoked by the presentation of a familiar stimulus (during retrieval) distinguishes stimuli that will be successfully recollected from stimuli that will not be recollected. At the same time, neuronal activity also predicts whether the stimulus is novel or familiar (familiarity). The response was strongest for stimuli that are both recollected and recognized, intermediate for items that were only recognized and weakest for items that are novel. The responses to items which have been seen before but were not recognized as such (forgotten), as well as false positives (false memory) were larger than for truly novel items but less than for recognized items. In patients that do not have the capability for recall (only recognition) the difference between only recognized and also recollected was entirely abolished. Importantly, the ability to predict (by decoding) whether a stimulus is familiar is not influenced by whether the stimulus will later be recollected. We thus conclude, that human medial temporal lobe neurons contain information about both components of memory. These data support a continuous strength of memory model of MTL function: the stronger the neuronal response, the better the memory.

Publications

Tai, C-Y., Mysore, S.P., Chiu, C. and Schuman, E.M. (2007) Activity regulated N-cadherin endocytosis. *Neuron*, **54**:771-785.

Dieterich, D.C., Lee, J.J., Link, A.J., Graumann, J., Tirrell, D.A. and Schuman, E. M. (2007) Labeling, detection, and identification of newly synthesized proteomes with bioorthogonal non-canonical amino acid-tagging. *Nature Proto*. **2**:532-540.

Sutton, M.A. and Schuman, E.M. (2006) Local dendritic protein synthesis and plasticity. *Cell* **127**:49-57.

Schuman, E.M., Dynes, J.L and Steward, O. (2006) Synaptic regulation of translation of dendritic mRNAs. *J. Neurosci.* **26**:7143-7146.

Beatty, K., Liu, J. Dieterich, D.C., Schuman, E.M. and Tirrell, D.T. (2006) Fluorescence visualization of newly synthesized proteins in mammalian cells. *Agnewandte Chemie* **45**:7364-7367.

Schuman, E.M. and Seeburg, P.H. (2006) Signalling mechanisms. *Curr. Op. Neurobiol.* **64**:116-131.

Bingol, B. and Schuman, E.M. (2006) Activity-dependent dynamics and sequestration of the proteasome in dendritic spines. *Nature* **441**:1144-1148.

Dieterich, D.C., Link, A.J., Graumann, J., Tirrell, D.T. and Schuman, E.M. (2006) Selective identification of newly synthesized proteins in mammalian cells using bioorthogonal non-canonical amino acid-tagging (BONCAT). *Proc. Natl. Acad. Sci. USA* **103**:9482-9487.

Sutton, M.A., Ito, H., Cressy, P., Kempf, C., Woo, J. and Schuman, E.M. (2006) Miniature synaptic transmission stabilizes synaptic function via tonic suppression of local dendritic protein synthesis. *Cell* **125**:785-799.

Rutishauser, U., Mamelak, A.N. and Schuman, E.M. (2006) Online detection and sorting of extracellularly recorded action potentials in human medial temporal lob recordings, *in vivo*. *J. Neurosci. Meths*. **154**:204-224.

Rutishauser, U., Mamelak, A.N., and Schuman, E.M. (2006) Learning, novelty and familiarity detection in single neurons of the human hippocampus-amygdala complex. *Neuron* **49**:805-813.

Tai, H.C. and Schuman, E.M. (2006) MicroRNAs reach out into dendrites. *Curr. Biol.* **16**:R121-123.

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Summary: While we continue to examine the dynamic/adaptive nature of human visual perception - including its crossmodal, representational, sensory-motor, developmental, emotional, and neurophysiological aspects - we put more emphasis on the ERATO (Exploratory Research for Advanced Technology) Shimojo "Implicit Brain Functions" project (supported by JST, Japan Science and Technology Corporation, officially started in October '04), with its emphasis on implicit cognitive processes, emotional decision making, and their neural correlates. Vigorous collaborations are conducted among our psychophysics laboratory here and the ERATO Japan site located at NTT Communication Science Laboratories, Atsugi, Kanagawa, Japan, as well as Harvard MGH, Boston University, Gordon College London, National Academy of Science Austria, and Decode Inc. Germany.

Using a variety of methods including eye tracking, high-density EEG, fMRI and MEG, we examine how exactly peripheral sensory stimuli, neural activity in the sensory cortex, and the mental experience of perception are related to each other. As for objectives of the ERATO project, we aim to understand implicit, as opposed to explicit or conscious, somatic and neural processes which lead to, and thus predict, conscious emotional decisions such as preference. As a more overarching general theme including both the perceptual psychophysics and the ERATO studies of emotional decision making, we are in particular interested in the intriguing interactions between *predictive* processes (prior to and thus predicting the mental event or behavior) and *postdictive* processes (posterior) to understand conscious experience of perception, sensory-motor learning, memory, and emotional decision modulated by internal and external factors. There are also a couple of technical developments in the project, i.e., fMRI-based neural conditioning and a video-based auditory-visual data collection/analysis system.

In addition, we have some purely theoretical meta-analysis on human color perception.

(1) We continue our work applying TMS (Transcranial Magnetic Stimulation) to the visual cortex of alert normal subjects, to reveal neural mechanisms underlying conscious visual perceptual experience. In our earlier finding of TMS-triggered "visual replay" effect, we had demonstrated that when a simple visual stimulus is presented followed by a dual-pulse TMS (which typically yields an illusory light field in the contralateral visual field, called "phosphene," the stimulus "reappears" perceptually, mainly within the area of phosphene. In our new study, we found that (a) natural scenes often generate a vivid perceptual replay, (b) the phenomenology of replay varies across observers, from a photopic to filling-in-like to contours alone, and (c) the replay often goes beyond the spatial range of phosphene (e.g., bilateral, symmetrical percepts). The results revealed new aspects of visual cortical traces and dynamics that lead to conscious perception. In yet another study we applied TMS to saccade mislocalization phenomenon, and provided the first direct evidence against any account based on the neural delay from the retina to the cortex. We showed that TMS-triggered phosphene, which would not have the neural delay that visual stimuli have, yet undergoes an equally large magnitude of saccade mislocalization.

(2) It is a commonsense knowledge in a daily life that our cognitive interpretation of own behavior makes a difference in future behavior, i.e., learning. Would this be still true in earliest level of sensory-motor learning such as saccade adaptation, and what kind of knowledge, strictly speaking, matters? In the saccade adaptation paradigm, an artificially created constant "retinal error" after a saccade to a visual target repeated over trials leads to modification of saccade amplitude accordingly. We asked whether or not (a) the subject's awareness of presence/absence of the corrective saccade; and (b) own cognitive interpretation of the second saccade as "corrective," matters for the occurrence of adaptive change. The results decisively indicated that none of these (a, b) matters – instead, mere presence of the second saccade itself (regardless of awareness or cognitive interpretation) exclusively matters for adaptation to occur. This really challenges the mainstream interpretation of saccade adaptation mechanism.

(3) The position of a flash presented during a smooth pursuit eye movement is mislocalized in the direction of the pursuit ("pursuit mislocalization"). We found that spatial contexts, such as the presence of an object ("obstacle") in the trajectory of mislocalization dramatically changed the amount of mislocalization as well as perceived spatial configurations. Moreover, the critical time period of the obstacle for such a spatial

reorganization is not before the presentation of a flash, not even during the flash, but rather immediately after it. This result strongly argues for the presence of some *postdictive* process that reorganizes the percept spatio-temporally before it reaches consciousness.

(4) How is a motion percept be related to each static snapshots is a classical problem? Our findings suggest that the moving stimulus is initially perceived as a time series of discrete potentially isolatable frames; later failures to perceive change suggests that over time, the stimulus begins to be perceived as a single, indivisible gestalt integrated over space as well as time.

(5) We found a new color spreading effect in which the foveal color is "filling-out" towards the periphery beyond luminance-defined edges after the eyes fixated for several seconds. Our psychophysical results indicate that color filling can be governed by a host of visual cues outside the realm of first-order color and brightness, via their impact on perceptual surface segmentation and segregation. Thus, brain compensate for low signal-to-noise ratios in the periphery by utilizing the foveal signals, to attain a more consistent scene interpretation.

(6) As a part of the ERATO project on visual preference decision, we examined novelty and familiarity as potential factors for attractiveness/ preference. We paired a new and old stimuli (within the same category, such as faces, natural scenes, or geometric shapes) in a two-alternative, forced-choice preference-decision paradigm. Surprisingly, faces and natural scenes behaved in opposite ways - familiarity won more and more across trials (with an identical old stimulus) in faces, whereas novelty won cumulatively in natural scenes. The results with geometric shapes were somewhere in between. The results indicate involvement of earlier sensory and memory factors in preference decision.

(7) As a part of the ERATO project, we examined fMRI signals in response to a more preferable face vs. a less preferable one (in collaboration with J. O'Doherty's and R. Adolph's laboratories). As a result, we found some early activation of a subcortical area (nucleus accumbens) with late activation of a more prefrontal area. The early activation was task-independent, thus reflecting automatic, bottom-up emotional activation.

(8) We compared reward and punishment avoidance in fMRI (in a collaboration with J. O'Doherty's laboratory). While obtaining a reward-elicited activity in classical reward structures, avoidance of punishment did not elicit significant activity in those areas. These data suggest that reward and avoidance conditioning are very distinct processes despite their common behavior-reinforcing properties. In another study (in a collaboration with J. O'Doherty's and R. Adolph's), we found a common neural pathway between juice and monetary award in the normal human.

(9) Neural feedback is a biofeedback technique where neural activity of a particular interest (such as burst-like precursor of epileptic seizure) is visualized and feedback to the patient in real-time, to assist improving the medical status. This has been mainly based on EEG signals, and only few successful reports are available with fMRI. We aimed "neural conditioning" with fMRI (in a collaboration with J. O'Doherty's laboratory), where instead of giving real-time feedback we only give a reward when the subject was successful in increasing (or suppressing) neural activity in ROI. With aid of the classical shaping technique, we were successful in differentially conditioning hand and feet movement areas in the motor cortex without allowing the subject to actually move the body parts. The results potentially have enormous impacts on both basic laboratory research in the normal alert human brain, as well as clinical applications such as anti-depression treatment.

(10) Stationary flashes shown between the presentation of an apparent motion stimulus are mislocalized in the direction of apparent motion (visual rabbit illusion, Geldard 1976). This perceptual displacement depends on the position of the last flash, suggesting that a "backward (postdictive) reconstruction" is taking place in perception. We investigated the neural correlates with a high-density EEG system (ERP), and found three distinctive stages, i.e., an early sensory stage, a motion stage, and a decision stage, the last of which was observed mainly in the frontal electrodes and after 240 ms from the last visual stimulus, thus postdictive in nature.

(11) Some types of perceptual learning are characterized by their self-organizing and location- and eye-specific properties, thus letting researchers speculating on the early visual cortex as the locus of neural changes. We made fMRI scanning while monitoring the subject's sleep with simultaneous EEG recording after intensive texture-segregation training. We found a significant change in BOLD signals in the primary visual cortex in the learning condition, whereas no such change in the control condition. Thus, the result indicates the neural correlates of perceptual learning in the very early visual cortex.

(12) Implicit non-verbal communication not only assists explicit communication but rather provides the basis for it. To fully understand, we need an inclusive behavioral recording system which can record both voices and bodily gestures of communicators simultaneously, and make it easy for researchers to analyze both interpersonal and intrapersonal synchrony or coherence. Based on MATAB codes, we have created such a system and are preliminarily analyzing data of verbal and non-verbal communication.

(13) We compared color modulation of bare skin color which signals emotional change, and sensitivity of the human color system in the relevant middle-wavelength range. We then came up with a surprising conclusion that evolution of primate color perception is at least under influence of socio-emotional signaling via skin color modulation in the others. We further analyze the relationship between evolution of bare skin and its visibility on one hand, and that of color perception on the other hand.

317. Spatial context confines and distorts undergoing pursuit mislocalization

Yasuki Noguchi, Shinsuke Shimojo*

The position of a flash presented during a smooth pursuit eye movement is mislocalized in the direction of the pursuit. This has been explained by a temporal mismatch between the slow visual processing of the flash and fast efferent signals for present eye position. Here we tested whether spatial context would also influence the perceived position of the flash. We put various continuously-lit objects (walls) between the veridical flash location and position where the flash would normally be mislocalized. Walls significantly reduced the mislocalization of the flash, largely preventing the flash from being mislocalized beyond the wall. No change in eye movements was observed that would reduce the mislocalization. When the wall was shortened or had a hole in its center, the number of trials in which the flash was mislocalized beyond the wall increased, but in those trials, the flash was vertically shortened as if cut off or funneled by the wall. The wall also induced color interactions – a red wall made a green flash appear yellowish if it was in the path of mislocalization. Finally, the critical time period for the presentation of the wall was found to be after the disappearance of the flash. These results indicate that features of the flash in pursuit-induced mislocalization are postdictively determined using broad spatial and temporal integration windows, which provides a new insight as to why the afferent signals of the flash are so delayed compared to the efferent eye-position signals.

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318. Familiarity vs. novelty principles for preference

Eiko Shimojo, Junghyun Park, Lauren LeBon, Stephen Schleim, Shinsuke Shimojo*

Understanding preference decision-making is a challenging problem because the underlying process is partly implicit and dependent on context, including past experience. There is evidence for both familiarity and novelty as a critical factor for preference decision making in adults and infants. To resolve this seeming contradiction, we examined cumulative effects of exposure in different categories of object.

Faces, geometric figures, and natural scenes were used separately in three blocks in a random order. Each block has eight sets of trials, in each of which 26 pairs of within category images were presented sequentially, and for each pair the participant judged relative preference in a seven-point scale. In each set of trials, one image was repeatedly presented at randomized location (L or R), paired with a new image each time. Thus except for the first trial, the pair/choice was always old vs. new.

When probability of preference for the old stimulus was plotted against trial #, faces showed a steady increase, thus a general tendency towards familiarity preference. There is no tendency of novelty preference through out the face set. On the other hand, natural scenes showed a quick decrease with saturation, a tendency of novelty in general. Geometric figures stayed neutral over trials, thus no strong bias either way. The overall pattern of results points to a possibility that the familiarity and the novelty principles are segregated across object categories. Different social/ecological significances may partly account for it.

References

Berlyne, D.E. (1970) Novelty, complexity, and hedonic value. *Percept. Psychophys*. **8**:279-286.

Bornstein, R.F. (1989) Exposure and affect: Overview and meta-analysis of research, 1968-1987. *Psychol. Bull.* **106**:265-289.

Kail, R.V. Jr. and Freeman, H.R. (1973) Sequence redundancy, rating dimensions, and the exposure effect. *Mem. Cognit.* **1**:454-458.

Meltzoff, A.N. and Borton, R.W. (1979) Intermodal matching by human neonates. *Nature* **282**:403-404.

Stang, D.J. and O'Connell, E.J. (1974) The computer as experimenter in social psychological research. *Behav. Res. Meth. Instru*. **6**:223-231.

Vessel, E.A., Biederman, I. and Cohen, M. (2004) Parahippocampal fMRI activity is modulated by scene type. Poster presented at the Vision Science Society Conference in Sarasota, FL.

Van Kreveld, D. (1972) Exposure, satiation, and stimulus discriminability. *J. Pers. Soc. Psychol*. **21**:270-280.

Zajonc, R.B. (1968) Attitudinal effects of mere exposure. *J. Pers. Soc. Psycho*. Mon Suppl **9**:1-27.

Zajonc, R.B., Shaver, P., Tavris, C. and Van Kreveld, D. (1972) *J. Pers. Soc. Psycho*. **221**:270-280.

319. On the possible relationship between orientation and choice

Harald Stogbauer, Simone Wehling, Joydeep Bhattacharya*, Shinsuke Shimojo*

Making preferential choices is a fundamental aspect of human life. Research efforts, so far, are heavily concentrated on explaining the choice by exogenous features of the stimuli, whereas little is known about the possible role of endogenous components such as orientation responses. This latter issue is extremely relevant when the features of the possible choices are very close.

In this study, we monitored the gaze of ten observers in five two-alternative-forced-choice tasks (2AFC) where they were asked to choose a face which was more: (i) attractive (ATTR); (ii) sympathetic (LIKE); (iii) unfriendly (DISL); and (iv) round (ROUN). An additional ATTR task was made with abstract pictures (ABST). The stimuli (faces or abstract) were matched according to an independent rating. Different gaze features, such as dwell times, number of fixations, likelihood of first fixation on the choice or the left stimulus, were analyzed.

General properties of 2AFC: Two significant features are common to all tasks: (1) a shorter first dwell time (Friedman, $p<0.001$); and (ii) an overall longer dwell time on the chosen stimulus (paired t test, $p<0.0001$).

Task differences: We found for ABST trials three features (shorter first, second last and total dwell times) that allowed their classification with a 56% accuracy (by a chance level of 20%). Among the face tasks, only the first dwell times showed significant differences (Friedman, $p<0.003$), in which ROUN exhibits the shortest dwell and separates from ATTR, LIKE and DISL.

These results indicate an overall association, albeit weak, between orientation and choice. The next step is to investigate the directional information of this association, i.e., orientation influences choice or vice versa; although this study does not conclusively support the former, the next step must be the extension to imaging techniques.

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320. Neural correlates of a saltation illusion

Harald Stogbauer, Virginie van Wassenhove, Shinsuke Shimojo

Stationary flashes shown before the presentation of an apparent motion stimulus are mislocalized in the direction of apparent motion (visual rabbit illusion, Geldard 1976). This perceptual displacement depends on the position of the last flash suggesting that a "backward reconstruction" is taking place in perception. Here, participants were tested on a visual rabbit paradigm while being recorded under electroencephalography (EEG). The stimuli consisted of three flashes: the first and third flash were 4 degrees apart (either to the left or right) and 25 degrees away from fixation. The second flash was shown either at the same location as the first flash (RAB) or at a location between the first and third flashes (SAL). Psychophysical results show that participants are unable to discriminate between RAB and SAL conditions. The EEG study investigated the time course of cortical processing by contrasting the SAL and RAB conditions. First, event-related potentials (ERPs) in occipital electrodes showed a significant amplitude difference between stimulus types shortly (~100 milliseconds) after the presentation of the second flash. At about 180 ms after presentation of the third flash, a significant amplitude difference was found in parietal electrodes. This ERP profile clustered RAB and SAL according to the perceptual outcome for the direction of motion (left or right). Starting at about 240 ms after presentation of the last flash, a significant difference between the RAB and SAL conditions was found independent of the direction of apparent motion. This difference was observed mainly in the frontal electrodes. The ERP results suggest three distinct stages in the processing of the visual rabbit illusion, a low-level feature stage in which the different locations for the second flash can be seen, a discrimination of motion direction stage and an "anti-illusion" stage which shows a categorization of RAB and SAL, despite no perceptual difference.

321. Corrective saccades drive saccadic adaptation independently of explicit interpretation of retinal error

Junghyun Park, Shinsuke Shimojo

Systematic displacements of saccade targets while the eye is in mid-flight lead to a gradual change in saccade amplitudes. A) What drives this saccadic adaptation: post-saccadic retinal error signals or secondary corrective saccades? B) How susceptible is this process to explicit knowledge regarding the source of the retinal error?

Participants (N=10) were tested in a 3x2 manipulation. A) Three conditions tested the effect of explicit cues signaling that post-saccadic retinal error was due to a target shift rather than saccade inaccuracy. In the conventional saccadic adaptation condition, the target spot simply stepped from 10º to 7º. In the color condition, the target shift was marked by a color change. In the bar condition, a long vertical bar was presented throughout the trial in-between the original and final target positions. B) Participants were either encouraged to make corrective saccades, following the target wherever it is; or prohibited from making corrective saccades, stopping wherever the first saccade landed. Each of the six condition combinations was tested on different days to prevent transfer of adaptation.

A) Saccadic adaptation occurred in all encouraged conditions and in about half of prohibited conditions where participants unconsciously and unwillingly made secondary saccades. Six participants successfully (>95% of trials) suppressed secondary saccades in >1 of the prohibited conditions. When secondary saccades were suppressed, saccadic adaptation was abolished despite the presence of retinal errors.

B) Explicit knowledge did not affect saccadic adaptation: secondary saccades to the new target location resulted in saccadic adaptation even in color and bar conditions where participants could clearly tell that the movement was to a new target location, rather than a correction of saccadic error.

These results suggest that the adaptive change in saccade amplitude involves implicit processes arising from the corrective saccade, and is independent of explicit knowledge of the source of visual error.

322. TMS induces detail-rich "instant replays" of natural images

Neil Halelamien, Daw-An Wu, Shinsuke Shimojo

We have previously demonstrated that transcranial magnetic stimulation (TMS) can cause the re-perception of recently presented visual stimuli.

Here we find that such replays can be experienced for natural scene stimuli, with a level of detail suggesting low-level rather than iconic representations.

TMS was administered using a Magstim dual-pulse setup sending pulses with 50 ms separation through a figure-8 coil. The coil position over occipital cortex was optimized to elicit vivid flashes of brightness (phosphenes) in a darkened room. We screened subjects to find those that perceived large, bright phosphenes near fixation. To

these subjects (N=7), we presented pictures of natural scenes and animals for 100 ms, followed by TMS at various ISIs. Subjects provided verbal descriptions, subjective ratings, and drew figures on the screen.

While TMS in a stable visual environment generally elicits phosphenes that are consistent across trials, colorless, and internally featureless, we found that TMS delivered shortly after image presentation led to the perception of clearly-defined forms that varied according to the content of the flashed image.

In this experiment, five out of the seven subjects reported percepts that drew from the preceding images. In the most vivid cases, these would appear to be nearly photographic repetitions in portions of the display. In other cases, subjects would perceive uniformly-filled, phosphene-like figures whose outlines matched, in detail, contours drawn from the preceding image (abstract by Wu *et. al*., describes double-blind validation of these effects). In early trials, subjects experienced the most vivid replay effects within narrow time windows, which varied from subject to subject between 150-250 ms. With continued stimulation, longer ISIs (as much as one second) became effective.

This study indicates that rich, detailed visual information remains encoded well after visual perception has ended, and that TMS can allow conscious access to these nascent low-level representations.

Reference

Halelamien, N., Wu, D.-A., Shimojo, J. (May, 2007) TMS induces detail-rich "instant replays" of natural images. Poster presentation, 7th Annual Meeting of the Vision Sciences Society, Sarasota, FL, USA.

223. TMS "instant replay" validated using novel doubleblind stimulation technique

Daw-An Wu, Neil Halelamien, Fumiko Hoeft, Shinsuke Shimojo*

Transcranial magnetic stimulation (TMS) can elicit the re-perception of recently presented visual stimuli, possibly reflecting access to nascent neural representations (Wu *et. al.* VSS 2001, 2002, 2004, Jolij and Lamme, VSS 2006). However, it is difficult to rule out the contributions of subject bias or confabulation. Here, we employ a custom-built stimulation device to validate the effect under psychologically double-blind conditions.

Our previous "instant replay" experiments employed the commonly used Magstim dual-pulse setup and figure-8 coil. In the modified setup, we take advantage of the fact that the optimal stimulus to stimulate visual cortex involves a lateral-to-medial orientation of the induced electric field. Thus, a horizontally oriented coil placed at the midline can bias the level of stimulation to the left or right hemisphere based on the direction of current flow.

We employ a custom switch and coils validated for their physical, physiological and psychological effects. It was found that subjects and experimenters could not discriminate between the forward and reversed current conditions.

We apply this technique to a natural image replay protocol (see abstract by Halelamien *et al*., for details). Subjects (N=4) were presented with mirror-symmetric images and then stimulated with TMS of a polarity determined by the computer. Subjects judged whether the replay percept was more vivid on the left or the right side, rated the level of imbalance on a 10-point analog scale, gave verbal reports and made drawings on the screen.

Ratings, verbal reports and drawings all showed clear and significant differences between trials employing different stimulus polarities. Stronger stimulation to the left hemisphere resulted in more vivid percepts in the right visual hemifield, and vice versa. This provides double-blind validation that cortical representations are being activated directly by TMS, and demonstrated the usefulness of this new device.

Reference

Wu *et al*., VSS 2001, 2002, 2004, Jolij and Lamme, VSS 2006.

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324. Brain activity related to consolidation of perceptual learning during sleep

Yuka Sasaki[1], Yuko Yotsumoto[1,2], Shinsuke Shimojo, Takeo Watanabe[2]

Recently, a large number of studies have suggested that sleep plays an important role in learning consolidation, although it is still controversial.

In the present study, we examined consolidation-related brain activity during sleep after perceptual training. We measured brain activity in sleep for about an hour using simultaneous EEG and fMRI technique. First, subjects underwent the adaptation protocol in which they slept inside the MRI scanner with electrode attached so that they familiarized themselves to fall asleep inside the scanner. Then, we measured BOLD signals in the visual cortex before and after training of a texture discrimination task. Perceptual learning of this task is known to be locationally-specific and may involve the primary visual cortex (V1) (Karni and Sagi, 1991). Stimulus targets were presented only in the upper left visual field. The post learning sleep session was conducted at the same night as the training. Relative BOLD changes in brain activity in both the trained and untrained regions in V1 during sleep compared to that in the wake period before the sleep onset were calculated. Sleep stages were determined by EEG signals using a standard method. Visual areas in each subject's brain had been localized in advance in a separate fMRI session. Results indicate that in the post learning sleep period, the relative brain change for the trained region in V1 was significantly different from the untrained region and for

the trained region during the sleep before the learning. In addition, before the training, there was no significant difference between relative brain changes in the untrained and trained regions in V1. These results indicate that sleep consolidation process occurs in a highly localized circuit specific to the location of a trained stimulus.

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325. Eccentricity compensator for log-polar sensor

*Sota Shimizu, Joel W. Burdick**

This paper aims at acquiring robust rotation, scale, and translation-invariant feature from a space-variant image by a fovea sensor. A proposed model of eccentricity compensator corrects deformation that occurs in a log-polar image when the fovea sensor is not centered at a target, that is, when eccentricity exists. An image simulator in discrete space remaps a compensated log-polar image using this model. This paper proposes Unreliable Feature Omission (UFO) that reduces local high frequency noise in the space-variant image using Discrete Wavelet Transform. It discards coefficients when they are regarded as unreliable based on digitized errors of the input image.

The first simulation mainly tests geometric performance of the compensator, in case without noise. This result shows the compensator performs well and its root mean square error (RMSE) changes only by up to 2.54[%] in condition of eccentricity within 34.08 [°]. The second simulation applies UFO to the log-polar image remapped by the compensator, taking its space-variant resolution into account. The result draws a conclusion that UFO performs better in case with more white Gaussian noise (WGN), even if the resolution of the compensated log-polar image is not isotropic.

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326. Simultaneous and independent acquisition of multisensory and unisensory associations

Aaron R Seitz[1], Robyn Kim[2], Virginie van Wassenhove, Ladan Shams[2]

In daily life, humans are constantly exposed to stimuli from multiple sensory modalities: seeing a car passing by is accompanied by sound, touching water in a fountain is both seen and heard but one may be watching a bird flying away while hearing the sound of a lawn mower. Hence, while some multisensory stimuli provide information about the same event in the world, others do not and the specific conditions underlying the integration of multisensory information into one unified percept are yet unknown. Here, we focus on the processes by which we learn and acquire knowledge in order to integrate information from multiple sensory modalities. For this, we introduce a novel audio-visual statistical learning procedure in which participants are passively exposed to a rapid serial presentation of arbitrary auditory, visual and audio-visual pairings. Being passively exposed to stimuli has previously been shown to be sufficient for robust learning. For our study, the set of stimuli consisted of synthetic auditory and visual displays which did not bear a specific representational (or semantic) value for the individuals (i.e., no prior knowledge). Participants also did not know prior to the exposure period that later on, they would be tested on their familiarity level with the presented pairings. Following the exposure period, participants were tested using a two-interval forced-choice procedure in which their degree of familiarity to the experienced audio-visual pairings was evaluated against novel audio-visual combinations drawn from the same stimulus set. Our results show that subjects do acquire knowledge of visual-visual, audio-audio and audio-visual stimulus associations. Additionally, the learning of these types of associations occurs in an independent manner, suggesting possible statistical regularities that are specific to multisensory stimuli. These results add to the body of research on implicit learning in which the statistical regularities of the sensory environment have been shown to shape learning and the neural plasticity during development.

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327. Speech perception at the interface of neurobiology and linguistics

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Speech perception consists of a set of computations that take as input continuously varying acoustic waveforms (or visual kinematics), and generate as output discrete representations that make contact with the lexical representations stored in long-term memory. Because the perceptual objects that are recognized by speech perception enter into subsequent linguistic computations, the format that is used for lexical representation and processing fundamentally constrains the (auditory and/or visual) speech perceptual processes. Consequently, theories of speech perception must, at some level, be tightly linked to theories of lexical representation. Minimally, speech perception must yield representations that smoothly and rapidly interface with stored lexical items. Adopting the perspective of David Marr (1982), we argue and provide neurobiological and psychophysical evidence for the following research program. First, at the implementation level, speech perception is a multi-time

resolution process, with perceptual analyses occurring concurrently on at least two time scales commensurate with (sub)segmental (~20-80 ms) and syllabic analyses (~150-300 ms). Second, at the algorithmic level, we suggest that speech perception proceeds on the basis of internal forward models and uses an 'analysis-by-synthesis' approach (Halle and Stevens 1959, 1962). From a neurobiological point of view, the perception of speech would thus entail a predictive mechanism that affords the generation of discrete representations. Third, at the computational level (in the sense of Marr), the theory of lexical representation that we adopt here is principally informed by phonological research and assumes that words are represented in the mental lexicon in terms of sequences of discrete segments composed of distinctive features. One important goal of the research program is thus to develop linking hypotheses between the putative neurobiological primitives (for example, temporal primitives) and those primitives that are derived from linguistic inquiry, in order to ultimately reach a biologically sensible and theoretically satisfying model of representation and computation in speech.

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Citation

Poeppel, D., Idsardi, W., van Wassenhove, V. (2007) *Phil. Trans. R. Soc. London B.* In press.

References

Halle, M. and Stevens, K.N. (1959) Analysis by synthesis. In: *Proc. of Seminar on Speech Compression and Processing* (ed. W. Wathen-Dunn and L.E. Woods), vol. 2, Paper D7.

Halle, M. and Stevens, K.N. (1962) *IEEE Transactions on Information Theory* **8**(2) 155-159.

Marr, D. (1982) *Vision*. W.H. Freeman, San Francisco, CA.

328. Auditory cortical plasticity in learning to discriminate modulation rate

*Virginie van Wassenhove, Srikantan S. Nagarajan**

The ability to discriminate the fine temporal structure of acoustic inputs is of crucial importance for auditory perception. Yet, very few studies of auditory perceptual learning have focused on training the capability to discriminate the temporal aspect of acoustic inputs. Here, we trained participants on a temporal discrimination task for three days (one hour per day) while they were recorded with magnetoencephalography (MEG). On the days preceding and following the training days, participants were tested on several control tasks testing for the transferring of learning. The training task was a temporal modulation rate task in which a base stimulus consisting of four tones separated by intervals of 200 ms had to be distinguished from a target stimulus consisting of four tones with intervals down to ~180 ms. Despite the short amount of training, participants' temporal sensitivity to auditory modulation rate significantly improved. After training, the perceptual improvements partly transferred to an interval discrimination task but not to a frequency discrimination task. These results suggest that it is specifically the discrimination of the temporal properties of the auditory stimuli which have improved. Learning to discriminate temporal modulation rates was accompanied by a systematic amplitude increase of the early magnetic auditory evoked responses (i.e., ~200 ms post-stimulus onset) to trained stimuli. Consistent with the psychophysical results, these indices of auditory cortex plasticity partially generalized to an interval discrimination but not to a frequency discrimination task. Specifically, auditory cortex plasticity associated with short-term perceptual learning was manifested as an enhancement of auditory cortical responses to trained acoustic features only in the trained task. Additionally, MRI-based source reconstructions in time-frequency space showed that plasticity was also manifested as an increased induced power in the high gamma frequency band (>70Hz). This increase was located in the inferior frontal cortex during performance in the trained task. All together, these results suggest that the functional plasticity observed here is an integrated product of bottom-up and top-down modulations interfacing in the auditory cortex.

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Citation

van Wassenhove, V. and Nagarajan, S.S. (2007) *J. Neurosci*. **27**(10) 2663-2672.

329. Hearing lips and seeing voices: How cortical areas supporting speech production mediate audiovisual speech perception

Jeremy I. Skipper[1], Virginie van Wassenhove, Howard C. Nusbaum[2], Steven L. Small[2]

In various social settings, observing a speaker's mouth can profoundly influence speech perception and improve speech intelligibility in a noisy environment. Additionally, listeners perceive an "illusory" "ta" when the video of a face producing /ka/ is dubbed onto an audio /pa/ (McGurk and MacDonald, 1976). Here, we show how cortical areas supporting speech production mediate this illusory percept and audiovisual (AV) speech perception more generally. Specifically, cortical activity during AV speech perception occurs in many of the same areas that are active during speech production. We find that different perceptions of the same syllable and the perception of different syllables are associated with different distributions of activity in frontal motor areas involved in speech production. Activity patterns in these frontal motor areas resulting from the illusory "ta" percept are more similar to the activity patterns evoked by AV/ta/ than they are to patterns evoked by AV/pa/ or AV/ka/. In contrast to the activity in frontal motor areas, stimulus-evoked activity for the illusory 'ta' in auditory and somatosensory areas and visual areas initially resembles activity evoked by AV/pa/ and AV/ka/, respectively. Ultimately, though, activity in these regions comes to resemble activity evoked

by AV/ta/. Together, these results suggest that AV speech elicits in the listener a motor plan for the production of the phoneme that the speaker might have been attempting to produce, and that feedback in the form of efference copy from the motor system ultimately influences the phonetic interpretation.

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Citation

Skipper, J.I., van Wassenhove, V., Nusbaum, H.C. and Small, S.L. (2007, EPub). *Cerebral Cortex, doi:10.1093/cercor/bhl147.*

Reference

McGurk, H. and McDonald, J. (1976) *Nature* **264-**746-747.

330. Temporal window of integration in auditory-visual speech perception

Virginie van Wassenhove, Ken W. Grant[1], David Poeppel[2]

Forty-three normal hearing participants were tested in two experiments, which focused on the temporal coincidence of auditory visual speech signals. Numerous reports suggest that visual speech inputs (i.e., seeing a talking face) can enhance or modify the perception of otherwise clear auditory speech signals. The mechanisms underlying auditory-visual (AV) speech integration are unclear but establishing the temporal constraints in AV speech integration is crucial for both models and neurophysiologic accounts of speech perception. In these experiments, audio recordings of /pa/ and /ba/ were dubbed onto video recordings of /ba/ or /ga/, respectively (ApVk, AbVg), in order to produce the illusory "fusion" percepts /ta/, or /da/ (McGurk and McDonald, 1976). In Experiment 1, an identification task using McGurk pairs with asynchronies ranging from −467 ms (auditory lead / visual lag) to +467 ms (auditory lag / visual lead) was conducted. Participants were tested using a three-alternative forced choice paradigm in which /ba/, /ga/, /da/ or /tha/ in the voiced conditions and /pa/, /ka/, or /ta/ in the voiceless conditions were possible answers. Each of these choices corresponded to an auditory-dominant response (i.e., the actual token presented in the auditory modality), a visual-dominant response (i.e., the actual token presented in the visual modality)or an illusory (i.e., auditory-visual fusion response), respectively. Results show that fusion responses were prevalent over temporal asynchronies from −30 ms to +170 ms and more robust for audio lags. In Experiment 2, simultaneity judgments for incongruent and congruent audiovisual tokens (AdVd, AtVt) were collected using a two alternative-forced choice paradigm where possible answers were 'simultaneous' or 'successive.' McGurk pairs were more readily judged as asynchronous than congruent pairs. Characteristics of the temporal window over which simultaneity *and* fusion responses were maximal were very similar, and suggest the existence of a ~200 ms duration asymmetric bimodal temporal integration window in AV speech processing.

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Citation

van Wassenhove, V., Grant, K.W. and Poeppel, D. (2007) *Neuropsychologia* **45**(3):598-607.

Reference

McGurk, H. and McDonald, J. (1976) *Nature* **264**:746-747.

331. Integrating subjective and objective measurements in gesture research

Rodrigo Segnini, Virginie van Wassenhove, Minae Okada*, Shinsuke Shimojo, Makio Kashino**

The implicit nature of gestures provides a window to evaluate mental processes not acknowledged by individuals. Most of what we know has been learned through the analysis of observable gestures and sounds in audiovisual recordings. While the analytical extraction of features should ideally be devoid of any evaluator's bias, certain aspects of this process, like the annotation of gesture types (e.g., phase and phrasing) and semantics, remain better achieved by visual inspection. How to integrate objective and subjective measurements to render new information easily interpretable for both? We have developed a framework for measuring cross-correlations among features automatically extracted from video and audio streams together with other features manually added into the pool of data. This framework is composed of readily available software components coordinated from within the MATLAB environment. For the analysis of visual data, functions from the Image Toolbox permit the measurement of size, quantity, and direction of activity (e.g., subject's movements). For the analysis of audio data (e.g., subject's voice), several custom routines extract standard features like fundamental and formant frequencies, amplitude, spectral centroid and roll off, among others; several types of data generated from within Praat can also be included at this stage. In order to visualize the most important cross correlations among segments of these features, the system outputs its results as an XML document, which can be loaded into the popular annotation tool ANVIL for integration with the hand-extracted gesture features. The result is then reloaded into MATLAB for a definitive analysis of co-occurrences between the objective and subjective measurements. This framework offers seamless integration of the various data generated by these widely used applications in the field of gesture research under a single-button operation - in most cases, this is all it takes to generate reports on synchronized video and/or audio files. Since all control codes are written in MATLAB, it can be scripted to generate cluster analysis across subjects and tasks.

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Citation

Segnini, R., van Wassenhove, V., Okada, M., Shimojo, S. and Kashino, M. (2007) [Abstract] *Presented at the 3rd Conference of The International Society for Gesture Studies*, Chicago, IL, USA.

332. Predictive coding in speech perception

Stefan J. Kiebel[1], Virginie van Wassenhove, Karl J. Friston[1], Katharina von Kriegstein[1]

Speech is a special case of multisensory perception in two respects. First, audio-visual speech is reliably correlated in both sensory modalities. Second, for many speech sounds, facial gestures precede the auditory input by dozens of milliseconds. If predictive coding extends over different sensory modalities, this timing difference could be used by the brain to predict the impending auditory input from observed visual gestures. A previous study showed that this might be the case: auditory speech processing was 'speeded up' for highly predictive visual speech syllables (van Wassenhove *et al.*, 2005) i.e., prior to auditory onset, the visual system provides information that is predictive for the auditory system. Two hypotheses can be derived from the literature: either integration (i) involves an early and automatic activation of motor areas to predict the auditory stream, or (ii) uses areas in the superior temporal sulcus (STS) to integrate visual and auditory information, and employs motor cortex only to resolve ambiguities in the input. In the present study we analyzed an event-related potential data set recorded during AV speech perception (using video stimuli) to adjudicate between these two claims. Source localization was performed using equivalent current dipoles and a Bayesian Expectation-Maximization algorithm. Three principle areas were involved in the processing of the visual input prior to the arrival of the auditory input (-200 ms to auditory onset), in both visual and AV stimuli. The activity spread from bilateral posterior superior temporal sulcus (STS/V), to bilateral primary motor cortex in the lip/tongue cortex (M1), and then to sources located around auditory cortex and STS (STS/A). The dipole strength in STS/V was similar for stimuli of different predictability (/pa/ vs. /ta/). However, in motor cortex and STS/A, /pa/ led to higher dipole strength than /ta/. This suggests that the motor cortex is used automatically as part of a forward model to predict auditory input: Motor activity is enhanced for highly predictive visual signals. The resulting predictions are forwarded to auditory association cortices (STS/A), where they initiate temporal facilitation of auditory processing.

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Citation

Kiebel, S.J., van Wassenhove, V., Friston, Karl J. and von Kriegstein, K. (2007) [Abstract] *Presented at the 13th Annual meeting of The Organization for Human Brain Mapping*, Chicago, IL.

Reference

van Wassenhove, V., Grant, K.W. and Poeppel, D. (2005) *Proc. Nat. Acad. Sci.* **102**(4):1181-1186.

333. Altering subjective time perception within and across senses

Virginie van Wassenhove, Dean Buonomano, Shinsuke Shimojo, Ladan Shams**

Subjective estimates of time generally reflect absolute time, but reliable biases are also observed. For instance, in a stream of standard events (of same duration and feature configuration), a stimulus differing along one perceptual dimension but of identical duration is perceived as longer than the standards. Here, we focus on time expansion in auditory, visual and auditory-visual contexts. Our experiments examined (i) the extent to which subjective duration depends on a feature change; (ii) the existence of the phenomenon across sensory modalities, and (iii) the contextual and physical parameters leading to a change of subjective duration. In a 2AFC design, participants judged whether a target (always fourth in a stream of five stimuli) was 'shorter' or 'longer' than the standards. In tests, the target changed in duration and feature properties; in controls, the target only varied in duration. A psychometric curve was derived from responses to the target whose duration spanned +/- 24% of the standard duration. In the auditory, visual and auditory-visual conditions, a looming signal in a stream of steady events induced an expansion of subjective time (i.e., a decrease in the point of subjective equality) whereas, a receding signals did not alter subjective duration. In the incongruent auditory-visual conditions, participants judged the duration of an auditory (visual) target, which was in all aspects identical to standards but concurrently presented with a visual (auditory) oddball. The subjective duration of visual targets were seldom lengthened by auditory inputs, whereas a visual input could either lengthened or shortened the perceived duration of an auditory target. Our results show that alterations in the subjective perception of time are independent of eye movements, and occur when there is no uncertainty about the position of the target in a stream of standards. These effects are likely governed by a combination of factors including stimulus features, contextual saliency, and multisensory interactions.

**University of California, Los Angeles, Los Angeles, CA*

Citation

van Wassenhove, V., Buonomano, D., Shimojo, S. and Shams, L. (2006) [Abstract] *Presented at the 36th Annual Meeting of the Society for Neurosciences*, Atlanta, USA, October 2006.

334. Audio-visual statistical learning

Ladan Shams[1], Aaron Seitz[2], Virginie van Wassenhove

Statistical learning theories suggest that people implicitly learn arbitrary stimulus-stimulus associations based solely on the statistics of inter-stimulus contingencies (Fiser and Aslin, 2001). Various studies have supported such learning in the visual, auditory and somatosensory modalities. To date, cross-modal statistical learning has not been investigated. Here, we present results of a novel audio-visual statistical learning procedure where participants are passively exposed to arbitrary audio-visual pairings (comprised of artificial/synthetic auditory and visual stimuli). Following this exposure period, the degree to which participants are familiar with the experienced audio-visual pairings is evaluated against novel audio-visual combinations drawn from the same stimulus set. The results of this comparison demonstrate the existence of audio-visual statistical learning. Additionally, we investigated whether audio-visual associations with an appropriate "gestalt" were learned more robustly than those with less appropriate relationships. We used a procedure in which visual objects disappeared into a 'visual abyss' and reappeared as new objects (Fiser and Aslin 2002). During each disappearance-to-reappearance interval, an upward or downward frequency-modulated auditory sweep was played. The Gestalt+ conditions consisted of either auditory downward-frequency sweeps paired with visual disappearances or auditory upward-frequency sweeps paired with visual appearances. The Gestalt- conditions consisted of either auditory downward-frequency sweeps paired with visual appearances or auditory upward-frequency sweeps paired with visual disappearances. Hence, Gestalt+ and Gestalt- conditions were opposite with respect to audio-visual meaningfulness. Under these conditions, subjects showed a greater familiarity for Gestalt+ than for Gestalt- audio-visual pairs. Our results suggest that audio-visual statistical learning occur naturally despite the absence of a task or of an explicit attentional engagement, for audio-visual stimuli that are spatio-temporally coincident. More importantly, the degree of learning partially depends on an appropriate gestalt relationship between the auditory and the visual events. These results raise interesting questions with respect to the plasticity underlying multisensory associations in the brain.

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Citation

Shams, L., Seitz, A. and van Wassenhove, V. (2006) [Abstract] *J. Vision*, 6(6), 152a, http://journalofvision.org/6/6/152/, doi:10.1167/6.6.152.

335. Statistical learning of auditory-visual associations

Virginie van Wassenhove, Aaron Seitz[1], Ladan Shams[2]

Statistical learning theories suggest that people implicitly learn arbitrary stimulus-stimulus associations based solely on the statistics of inter-stimulus contingencies (Fiser and Aslin, 2001). A number of studies have demonstrated the existence of implicit learning in the visual, the auditory and the somatosensory modalities but to date, multisensory statistical learning has not been reported. Here, we tested whether statistical learning could be observed for synthetic multisensory pairings, namely audiovisual pairs. Both the joint and the conditional probabilities of auditory-visual stimulus contingencies were tested in different groups of participants using a rapid-serial presentation (RSP) paradigm, in which stimuli were rapidly displayed one after the other with various inter-stimulus intervals. During the experiment, participants were first exposed to the stimuli which they were asked to observe passively (i.e., no response was expected). At this stage of the experiment, participants were not aware of the actual goal of the experiment, nor did they know that they would subsequently be tested on the frequency of the stimuli. In the second phase of the experiment, participants were presented with 'singles' (i.e., one auditory or one visual stimulus), 'doublets' (i.e., two auditory, two visual or one auditory and one visual stimuli) or quartets (two pairs of bimodal stimuli) in a two-interval forced choice paradigm. Participants were asked to determine the interval in which the stimulus was most frequently occurring during the exposure period – i.e., the frequency of the stimuli. In our analysis, we examined the effects of: (i) stimulus duration in the RSP procedure; (ii) joint probability; and (iii) conditional probability on statistical learning. Our results suggest that audio-visual statistical learning does occur naturally despite the absence of a task or of an explicit attentional engagement from the participants. Additionally, auditory-visual statistical learning was observed to be more efficient than auditory or visual learning across all stimulus presentation rates.

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Citation

van Wassenhove, V., Seitz, A.R. and Shams, L. (2006) [Abstract] *Presented at the 7th Annual meeting of the International Multisensory Research Forum*, Dublin, Ireland.

336. Direct instrumental conditioning of neural activity using fMRI derived reward feedback

Signe Bray[1], Shinsuke Shimojo, John P. O'Doherty[1,2]

Successful learning is often contingent on feedback. In instrumental conditioning, an animal or human learns to perform specific responses in order to obtain reward. Instrumental conditioning is often used by

behavioral psychologists in order to train an animal (or human) to produce a desired behavior. Shaping involves reinforcing those behaviors that in a step-wise fashion are successively closer to the desired behavior until the desired behavior is reached. Here, we aimed to extend this traditional approach in order to directly shape neural activity instead of overt behavior. To achieve this we scanned 22 human subjects with fMRI and performed image processing in parallel with acquisition. We delineated regions of interest (ROIs) in finger and toe motor/somatosensory regions, and used an instrumental shaping procedure to induce a regionally specific increase in activity by providing an explicit monetary reward to reinforce neural activity in the target areas. After training, we found a significant and regionally specific increase in activity in the ROI being rewarded (finger or toe) and a decrease in activity in the non-rewarded region. This demonstrates that instrumental conditioning procedures can be used to directly shape neural activity, even without the production of an overt behavioral response. This procedure offers an important alternative to traditional biofeedback based approaches, and may be useful in the development of future therapies for stroke and other brain disorders.

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[2]*Division of Humanities and Social Sciences*

337. Effects of eye position upon activity of neurons in macaque superior colliculus

Michael Campos, Anil Cherian, Mark A. Segraves**

We examined the activity of neurons in the deep layers of the superior colliculus of awake, behaving rhesus monkeys during the performance of standard oculomotor tasks as well as during self-guided eye movements made while viewing natural images. The standard tasks were used to characterize the activity of neurons based upon established criteria.

The natural viewing paradigm enabled the sampling of neuronal activity during saccades and fixations distributed over a wide range of eye positions. Two distinct aspects of eye movement behavior contributed to the modulation of firing activity in these neurons. The well-established influence of saccade amplitude and direction was strongest and most prevalent surrounding the time of the start of the saccade. However, the activity of these neurons was also affected by the orbital position of the eyes, and this effect was best observed during intervals of fixation. Many neurons were sensitive to both parameters and the directions of their saccade vector and eye position response fields tended to be aligned. The sample of neurons included visual, buildup, and burst activities, alone or in combination. All of these activity types were included in the subpopulation of neurons with significant eye position tuning, although position tuning was more common in neurons with buildup or burst activity and less common in neurons with visual activity. The presence of both eye position as well as saccade vector signals in the superior colliculus is likely important for its role in the planning and guidance of combined movements of the eyes and head.

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338. Neural representation of sequential states within and instructed task

*Michael Campos, Boris Breznen, Richard A. Andersen**

In the study of the neural basis of sensorimotor transformations, it has become clear that the brain does not always wait to sense external events and afterwards select the appropriate responses. If there are predictable regularities in the environment, the brain begins to anticipate the timing of instructional cues and the signals to execute a response, and even the consequences of actions. An organism's ability to anticipate events reveals an internal representation of the sequential progression of behavioral states within the context of the task being performed. Using the same eye movement tasks while recording neural data from two cortical ocuolomotor areas in the rhesus monkey, we found complementary spatial and sequential state representations of the Lateral Intraparietal Area (LIP) and the Supplementary Eye Field (SEF). While both areas encoded the position of eye movement targets, this spatial encoding was more consistently found in single neurons of LIP. In addition, the neurons of the SEF were found to collectively encode the progression of behavioral states of the task, with individual neurons detecting and/or anticipating different events or sets of events in the task or becoming tonically activated or depressed from one event to another and thus encoding states in an event-based manner. The entirety of responses from SEF was used to decode the current temporal position within the context of the task. Since LIP neurons were found to respond similarly when encoding an eye movement plan (saccade period) or the location of brightly flashed stimulus (cue period), the temporal information provided from SEF could be used to imply the significance of the spatial representation found in LIP.

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339. Selection of targets and active disregard of irrelevant information, in monkey LIP and SEF

Michael Campos, Boris Breznen, Richard A. Andersen

Using asynchronous presentations of a target and distracter in a saccade task, we believe we have uncovered two important components of the neural mechanism of target selection, including some evidence for the active disregard of irrelevant information.

We recorded single unit activity from cortical areas SEF and LIP in two monkeys during this variant on the memory-guided saccade task, in which a distracter stimulus (one of two green polygonal objects) was presented prior to the cue stimulus (small while dot), which was briefly flashed. The distracter persisted until just before the signal for the monkey to saccade to the remembered location of the spatial cue.

Many LIP neurons (26%, 31/120) showed spatially tuned responses to both the distracter and cue presentations, though the distracter response was transient (100%, 31/31), and the cue response was maintained (84%, 26/31) throughout the memory period. Thus, in agreement with many published findings, LIP responds transiently to all flashed stimuli, but selectively maintains representations of intended eye movement targets. Many SEF neurons respond in a still more selective manner, most (24%, 40/165) responding selectively only to the cue, and not responding to the distracter. Surprisingly, some (9%, 15/165) SEF neurons responded only to the distracter, and were silent during the presentation of the cue. These SEF responses could be thought to label the task relevance of the target and distracter stimuli. Another class of SEF neurons responded to both the cue and distracter, but both of these responses tended to be transient, perhaps marking the passage of time in an event-based manner (Campos *et al.*, 2005). Taken together, we find that, first, frontal cortex labels relevant and irrelevant stimuli, and second, parietal cortex selectively maintains the spatial representations required for planning behavior. These are important components of target selection when various stimuli are presented at different times.

340. Comparing neural representations of expected reward for food and money

Hackjin Kim[1], Shinsuke Shimojo, Ralph Adolphs[2], John P. O'Doherty[1]

Humans are capable of learning to predict rewards of many different types, including food (specifically juice reward) and money. Although many previous studies have examined neural responses during expectation of juice and money, no study has yet compared responses to both types of reinforcer directly within the same experiment, in the same subjects. As a consequence, it is not yet known whether there exists a region of the brain that commonly encodes expected reward for both types of reward, or whether expectations of food and money reward engage distinct and non-overlapping circuitry. Here, we used a simple variant of a reward-based action selection task, in which subjects performed actions in order to obtain both food and money rewards, focusing in particular on the orbitofrontal and medial prefrontal cortices, as these areas have previously been shown to be involved in encoding expected reward value for both food and money rewards. Each trial began with the presentation of a visual cue or discriminative stimulus (DS). The shape, color and brightness of the DS informed subjects of the specific type, valence and magnitude of outcome that would be subsequently delivered. Subjects had to respond immediately after seeing each DS in order to obtain the outcome, which was delivered four seconds later. We computed contrasts to detect regions showing increasing responses to increasing magnitude of reward separately for food and money rewards. We found that while vmPFC is involved in encoding expectations of both reinforcer types in a group level, in fact these representations are distinct in individual subject level. Our finding of adjacent but non-overlapping representations suggests that a single brain region does not code commonly for the value of these two reinforcer types. Thus a common utility, if it exists, must be computed by 'binding' these two distinct representations together, perhaps analogous to how color and motion signals may be computed in the visual system.

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341. Temporal dissociation of subcortical and cortical systems in social decision-making

Hackjin Kim, Ralph Adolphs*, John P. O'Doherty*, Shinsuke Shimojo*

We express behavioral preferences all the time, often without much deliberation, yet the processes that constitute this ability have remained elusive. One key difficulty is that such preference decisions are often too rapid for their components to be investigated with fMRI. In the present study, we examined hemodynamic responses recruited at sequential stages within individual trials in which subjects made choices between pairs of face stimuli to indicate which face out of each pair they preferred, by using a temporally extended decision-making (TED) paradigm, in which subjects' decision times are extended due to limited exposure times of faces and variable intervals between repetitions of face stimuli. Whereas the nucleus accumbens was activated early, consistent with a role in initial evaluation and preference formation, orbitofrontal cortex was activated at later stages of decision-making, suggesting a role for this region in linking value to behavioral choice. These patterns of activation were driven by the eventual choice outcomes, and were specifically engaged during preference decisions and not during non-preference related decisions such as by judging the physical roundness of the same face stimuli. These findings suggest the importance of different temporal components during preference decision making and indicate that these different components may map onto distinct neural structures.

**Division of Humanities and Social Sciences*

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42. Dynamical evolution of motion perception

Ryota Kanai, Bhavin R. Sheth, Shinsuke Shimojo*

Motion is defined as a sequence of positional changes over time. However, in perception, spatial position and motion dynamically interact with each other. This reciprocal interaction suggests that the perception of a

moving object itself may dynamically evolve following the onset of motion. Here, we show evidence that the percept of a moving object systematically changes over time. In experiments, we introduced a transient gap in the motion sequence or a brief change in some feature (e.g., color or shape) of an otherwise smoothly moving target stimulus. Observers were highly sensitive to the gap or transient change if it occurred soon after motion onset (< or -200 ms), but significantly less so if it occurred later (> or -300 ms). Our findings suggest that the moving stimulus is initially perceived as a time series of discrete potentially isolatable frames; later failures to perceive change suggests that over time, the stimulus begins to be perceived as a single, indivisible gestalt integrated over space as well as time, which could well be the signature of an emergent stable motion percept.

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Publication

Kanai R., Sheth, B.R. and Shimojo, S. (2007) *Vision Res*. **47**:937-945.

343. Discrete color filling beyond luminance gaps along perceptual surfaces

Ryota Kanai, Daw-An Wu, F.A. Verstraten, Shinsuke Shimojo*

Perceived color at a point in space is not determined simply by the color directly stimulating the corresponding retinal position. Surface color is informed by flanking edge signals, which also serve to inhibit the intrusion of signals from neighboring surfaces. Spatially continuous local interactions among color and luminance signals have been implicated in a propagation process often referred to as filling-in. Here, we report a phenomenon of discrete color filling whereby color jumps over luminance gaps filling into disconnected regions of the stimulus. This color filling is found to be blocked at boundaries defined by texture. The color filling is also highly specific to the elements belonging to a common perceptual surface, even when multiple surfaces are transparently overlaid. Our results indicate that color filling can be governed by a host of visual cues outside the realm of first-order color and brightness, via their impact on perceptual surface segmentation and segregation.

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Publication

Kanai R., Wu, D.-A., Verstraten, F.A.J., Shimojo, S. (2006) *J. Vision* **6**(12):1380-1395

344. Cross-task repetition amnesia: Impaired recall of RSVP targets held in memory for a secondary task

Mark R Nieuwenstein[1], Addie Johnson[2], Ryota Kanai, Sander Martens[2]

People often fail to select and encode the second of two targets presented within less than 500 ms in rapid serial visual presentation (RSVP), an effect known as the attentional blink. We investigated how report of the two targets is affected when one of them is maintained in working memory for a secondary, memory-search task. The results showed that report of either target was impaired when it was a member of the memory set relative to when it was not. This effect was independent of both the temporal interval separating the RSVP target from the presentation of the memory set and the interval separating the targets. We propose that the deficit in recall occurs because the association between a target and the memory-search task interferes with the formation of a new association between that target and the following RSVP task, with the result that observers may be biased to ascribe the target only to the memory set.

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Publication

Nieuwenstein, M.R., Johnson, A., Kanai, R. and Martens, S. (2006) *Acta Psychologica*: doi:10.1016/j.actpsy.2006.08.006

345. Planning and online control of goal directed movements when the eyes are 'relocated'

Anne-Marie Brouwer, Quoc C Vuong*, Ryota Kanai*

We investigated the effects of different viewpoints on remapping visuo-motor space, and whether remapping happens differently during the planning and the online control phase of goal-directed movements. Participants tapped targets on a monitor that was placed horizontally flat and flush with the table in front of them. They viewed the layout of the scene, including the monitor, and their hand, through video goggles attached to a camera. The camera could be moved along a semi-circle with the monitor as the circle's center. On each trial, the camera was randomly positioned at one of seven locations on the semi-circle (-90 to +90 degrees in 30 degree steps), always at eye height. The time needed to tap the target was quickest when the camera was approximately facing the participant and progressively increased when the camera was located more to the sides (Experiment 1). There was no effect of camera location on performance when participants only saw the static layout of the scene and were not allowed to see the scene or their hand during the movement (Experiment 2). By comparison, the dependency of performance on camera location increased when participants did not have information about the layout of the scene at the start of the trial, and could only perform remapping when their hand was visible during the movement (Experiment 3). These results indicate that visuo-motor remapping happens differently during the planning phase when only static information about the layout is available, and during the control phase when dynamic information about the moving hand is also available.

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Publication

Brouwer, A., Vuong, Q.C. and Kanai, R. (2006) *Exp. Brain Res*. **175**(3):499-513.

346. Stimulus flicker alters interocular grouping during binocular rivalry

Tomas H.J. Knapen, Chris, L.E. Paffen*, Ryota Kanai, Raymond van Ee*

When the two eyes are presented with sufficiently different stimuli, the stimuli will engage in binocular rivalry. During binocular rivalry, a subject's perceptual state alternates between awareness of the stimulus presented to the right eye and that presented to the left eye. There are instances in which competition is not eye-based, but instead takes place between stimulus features, as is the case in flicker and switch rivalry (F&S). Here we investigate another such instance, interocular grouping, using a Diaz-Caneja-type stimulus in conjunction with synchronous stimulus flicker. Our results indicate that stimulus flicker increases the total duration of interocularly bound percepts, and that this effect occurs for a range of temporal flicker frequencies. Furthermore, the use of contrast-inversion flicker causes a decrease of total dominance duration of the interocularly bound percepts. We argue that different flickering regimes can be used to differentially stimulate lower and higher levels of visual processing involved in binocular rivalry. We propose that the amount of interocularly combined pattern-completed percept can be regarded as a measure of the level at which binocular rivalry is resolved.

**Utrecht University, Utrecht, The Netherlands*

Publication

Knapen, T., Paffen, C., Kanai, R. and van Ee, R. (2007) *Vision Res*. **47**(1):1-7.

347. Flash suppression and flash facilitation in binocular rivalry: A dual role of adaptation

Jan W. Brascamp, Tomas H.J. Knapen*, Ryota Kanai, Raymond van Ee*, Albert V. van den Berg**

We studied how prior exposure (priming) affects processing of visual stimuli, by assessing dominance in binocular rivalry. We show that besides impairing a stimulus' dominance (flash suppression), priming can also facilitate it, a novel phenomenon we call flash facilitation. A prime's effect depends on its energy (power duration): strong primes suppress, weak primes facilitate. This applies to both eye-priming and pattern priming, suggesting a general mechanism. We argue that these observations reflect adaptation of the primed percept. Although suppressive effects are generally attributed to adaptation, facilitatory effects are normally considered unrelated. Our findings, however, indicate that weak adaptation enhances dominance, whereas strong adaptation suppresses. A model that exhibits this property explains our data, as well as perceptual memory for ambiguous figures, providing a common framework for both phenomena. These findings indicate that adaptation in general has a dual nature, and plays both a suppressive and a facilitatory role in perception.

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Publication

Brascamp, J.W., Knapen, T.H.J., Kanai, R. , van Ee, R. and van den Berg, A.V. (2007) *J. Vision*. In press.

348. Recognizing the forest, but not the trees: an effect of colour on scene perception and recognition

Tanja C.W. Nijboer, Ryota Kanai, Edward H.F. de Haan*, Maarten J. van der Smagt**

Colour has been shown to facilitate the recognition of scene-images, but only when these images contain natural scenes, for which colour is 'diagnostic.' Here we investigate whether colour can also facilitate memory for scene images, and whether this would hold for natural scenes in particular. In the first experiment participants first studied a set of colour and greyscale natural and man-made scene images. Next, the same images were presented, randomly mixed with a different set. Participants were asked to indicate whether they had seen the images during the study phase. Surprisingly, performance was better for greyscale than for coloured images, and this difference is due to the higher false alarm rate for both natural and man-made coloured scenes. We hypothesized that this increase in false alarm rate was due to a shift from scrutinizing details of the image to recognition of the gist of the (coloured) image. A second experiment, utilizing images without a nameable gist, confirmed this hypothesis as participants now performed equally on greyscale and coloured images. In the final experiment we specifically targeted the more detail-based perception and recognition for greyscale images versus the more gist-based perception and recognition for coloured images with a change detection paradigm. The results show that changes to images are detected faster when image-pairs were presented in greyscale than in colour. This counterintuitive result held for both natural and man-made scenes (but not for scenes without nameable gist) and thus corroborates the shift from more detailed processing of images in greyscale to more gist-based processing of coloured images.

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Publication

Nijboer, T.C.W., Kanai, R. and de Haan, E.H.F. and van der Smagt, M.J. (2007) *Conscious. & Cogn*. In press.

349. Perceiving-the-present and a systematization of illusions

Mark A. Changizi, Andrew Hsieh, Ryota Kanai, Romi Nijhawan, Shinsuke Shimojo*

Over the history of the study of visual perception there has been great success at discovering countless visual illusions. There has been less success in organizing the overwhelming variety of illusions into empirical generalizations (much less explaining them all via a

unifying theory). Here we show that it is possible to systematically organize more than 50 kinds of illusion into a 7 by 4 matrix of 28 classes. In particular, we demonstrate that: (1) smaller sizes; (2) slower speeds; (3) greater luminance contrast; (4) farther distance; (5) lower eccentricity; (6) greater proximity to the vanishing point; and (7) greater proximity to the focus of expansion all tend to have similar perceptual effects, namely to (A) increase perceived size; (B) increase perceived speed; (C) decrease perceived luminance contrast; and (D) decrease perceived distance. The detection of these empirical regularities was motivated by a hypothesis, called "perceiving-the-present," that the visual system possesses mechanisms for compensating neural delay during forward motion, and we show how this hypothesis predicts the empirical regularity.

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Publication

Changizi, M., Hsieh, A., Nijhawan, R., Kanai, R. and Shimojo, S. (2007) *Cogn. Sci.* In press.

350. Perceptual memory of ambiguous figures survives spontaneous perceptual alternations

Jan Brascamp, Tomas Knapen*, Ryota Kanai, Raymond van Ee*, Albert van den Berg**

Introduction: The visual system displays memory for perception of ambiguous figures: Perceptual dominance following an interruption depends on the last dominant percept before the interruption. A common interpretation of this phenomenon is that the last preceding percept leaves some form of memory trace, which in turn determines perceptual choice following the interruption. Here we show that, on the contrary, the last preceding percept has no special importance in determining the content of memory. Rather, perceptual choice is determined by a minute-scale history of prior perception, which includes, but is not limited to, the last preceding percept.

Methods: We establish the content of memory by intermittently presenting an ambiguous stimulus until a subject consistently perceives the same percept for eight presentations in a row: robust perceptual memory. Then we leave the stimulus on continuously, causing spontaneous perceptual alternations. Finally, we probe the content of memory following spontaneous alternations by assessing dominance during a renewed period of intermittent presentation.

Results: Perceptual choice following spontaneous alternations is influenced by the last percept during the alternations, but more strongly by the content of memory prior to the alternations. This long-lasting memory trace survives up to about one minute, or more than four perceptual alternations. This is evidence of unexpectedly complex memory behavior in ambiguous perception, indicating information integration over prolonged periods of time rather than straightforward storage of a single percept.

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Publications

Brascamp, J.W., Knapen, T.H.J., Kanai, R. van Ee, R. and van den Berg, A.V. (2007) Flash suppression and flash facilitation in binocular rivalry: A dual role of adaptation. *J. Vision*. In press.

Brouwer, A., Vuong, Q.C. and Kanai, R. (2006) Planning and online control of goal directed movements when the eyes are 'relocated.' *Exp. Brain Res*. **175**(3):499-513.

Brungart, D.S., van Wassenhove, V., Brandewie, Romigh, E. and Griffin, R.E. (2007) The effects of temporal acceleration and deceleration on auditory-visual speech perception. Proceedings of the International Conference on Auditory-Visual Speech Processing (AVSP07), Kasteel Groenendael, Hilvarenbeek, Netherlands.

Campos, M., Cherian, A. and Segraves, M.A. (2006) Effects of eye position upon activity of neurons in macaque superior colliculus. *J. Neurophysiol.* **95**:505-526. First published online 9/28/2005.

Changizi, M.A. (2006) The optimal human ventral stream from estimates of the complexity of visual objects. *Biol. Cybernet.* **94**:415-426.

Kanai R., Wu, D.-A., Verstraten, F.A.J. and Shimojo, S. (2006) Discrete color filling beyond luminance gaps along perceptual surfaces. *J. Vision* **6**(12):1380-1395.

Kanai R., Sheth, B.R. and Shimojo, S. (2007) Dynamical evolution of motion perception. *Vision Res*. **47**:937-945.

Knapen, T., Paffen, C., Kanai, R. and van Ee, R. (2007) Stimulus flicker alters interocular grouping during binocular rivalry. *Vision Res*. **47**(1):1-7.

Nieman, D., Nijhawan, R., Khurana, B. and Shimojo, S. (2006) Cyclopean flash lag illusion. *Vision Res*. **46**:3909-3914.

Nieuwenstein, M.R., Johnson, A., Kanai, R. and Martens, S. (2006) Cross-task repetition amnesia: Impaired recall of RSVP targets held in memory for a secondary task. *Acta Psychologica*: doi:10.1016/j.actpsy.2006.08.006.

Shimizu, S. (2007) A model of wide-angle foveation for all-purpose use of the Japan Society of Mechanical Engineers (in Japanese) Vol. 073, No.731, C（2007.7).

Shimojo, S. (2006) Active, as opposed to passive, aspect of action – concerning neural mechanisms underlying decision making. *J. Japan Soc. Mech. Eng.* **109**:1049:261-264, 2006 (in Japanese).

Simion, C. and Shimojo, S. (2007**)** Interrupting the cascade – orienting contributes to decision making even in the absence of visual stimulation. *Percept. Psychophy*. **69**(4):591-595.

Skipper, J.I., van Wassenhove, V., Nusbaum, H.C. and Small, S.L. (2007, Epub) Hearing lips and seeing voices: How cortical areas supporting speech production mediate audiovisual speech perception. *Cerebral Cortex* doi:10.1093/cercor/bhl147.

van Wassenhove, V. (2007) Analysis-by-synthesis in auditory-visual speech perception: Sensori-motor interfacing. Proceedings of the 16th International Congress of Phonetic Sciences, Säärbrucken, Germany.

van Wassenhove, V., Grant, K.W. and Poeppel, D. (2007) Temporal window of integration in bimodal speech. *Neuropsychologia* **45**(3):598-607.

van Wassenhove, V. and Nagarajan, S.S. (2007) Auditory-cortical plasticity in learning to discriminate modulation rate. *J. Neurosci*. **27**(10):2663-2672.

Yulmetyev, R.M., Yulmetyeva, D.G., Shimojo, S., Khusaenova, E.V. and Bhattacharya, J. (2007) Strong memory in time series of human magnetoencephalograms can identify photosensitive epilepsy. *J. Exp. Theoret. Physics* **104**:644-650.

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Postdoctoral Scholar: Evgueniy Lubenov
Graduate Students: Ming Gu, Casimir Wierzynski

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Summary: Our research focuses on the study of learning and memory formation in freely behaving animals at the level of networks of neurons. Previous research has shown that the hippocampus is critical for the formation of long-term declarative memories, and that this hippocampal involvement is time-limited. The current predominant conjecture is that memories are gradually established across distributed neocortical networks through the interactions between cortical and hippocampal circuits.

However, the direct experimental investigation of these interactions has been difficult since, until recently, simultaneous chronic recordings from large numbers of well-isolated single neurons were not technically feasible. These experiments became possible with the advent of the technique of chronic multi-area tetrode recordings in freely behaving rodents. Using this technique we monitor the simultaneous activity of large numbers of cortical and hippocampal cells during the acquisition and performance of memory tasks, as well as during the sleep periods preceding and following experience.

Our research efforts focus on analyzing the structure of cortico-hippocampal interactions in the different brain states and on characterizing how this structure is modulated by behavior; how it evolves throughout the learning process; and what it reflects about the intrinsic organization of memory processing at the level of networks of neurons. Our experimental work is complemented by theoretical studies of network models and the development of tools for the analysis of multi-neuronal data.

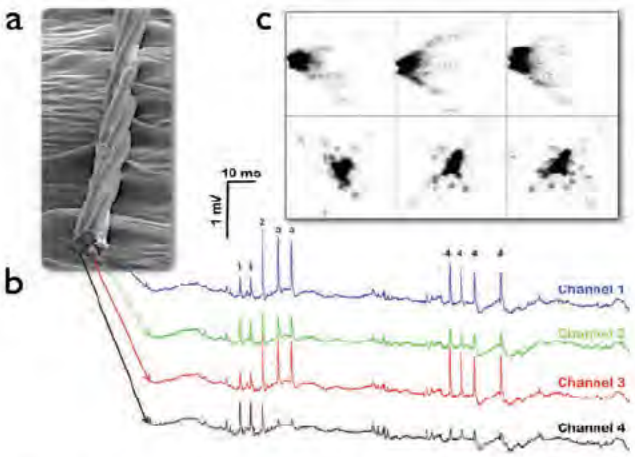

Figure 1: *Hippocampal Tetrode Recordings.* (a) Scanning EM image of a tetrode, which consists of four microwires twisted together to form a single recording probe. (b) Because of the spatial separation between the wire tips and the signal sources, each action potential is recorded simultaneously on all four wires, but with different amplitudes. This enables isolating the activity of multiple neurons in the vicinity of the tetrode tip, through a process analogous to triangulation. (c) The six 2-D projections of the Hadamard transformation of the 4-D space of amplitudes. Individual cells correspond to clusters in this space.

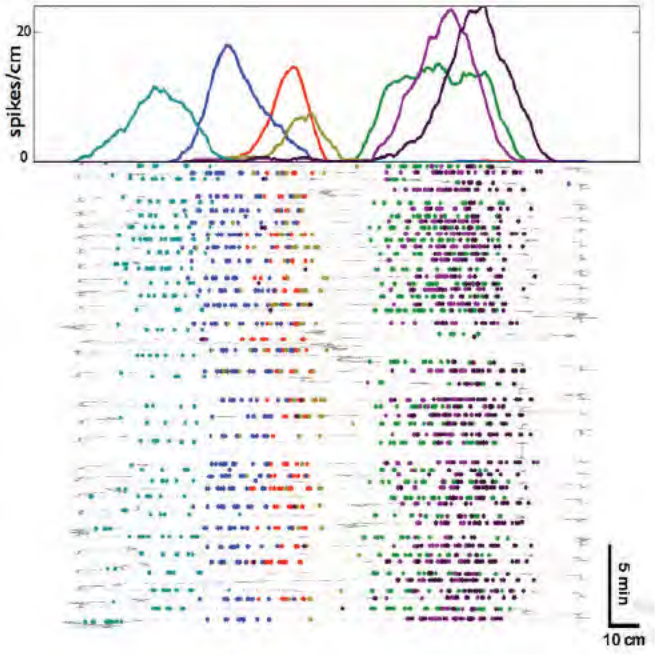

Figure 2: *Place fields.* The bottom panel shows that position of a rat in gray as a function of time (total time 28 minutes). The rat runs on a linear track back and forth and the positions and times of the spikes of seven simultaneously recorded place cells are shown in different colors. The top panel displays histograms of all spikes of each cell as a function of position along the track. Each of these cells ("place cells") fires in a specific location on the track ("place field"). The histograms are binned at 1cm and smoothed with a Gaussian with σ =10 cm.

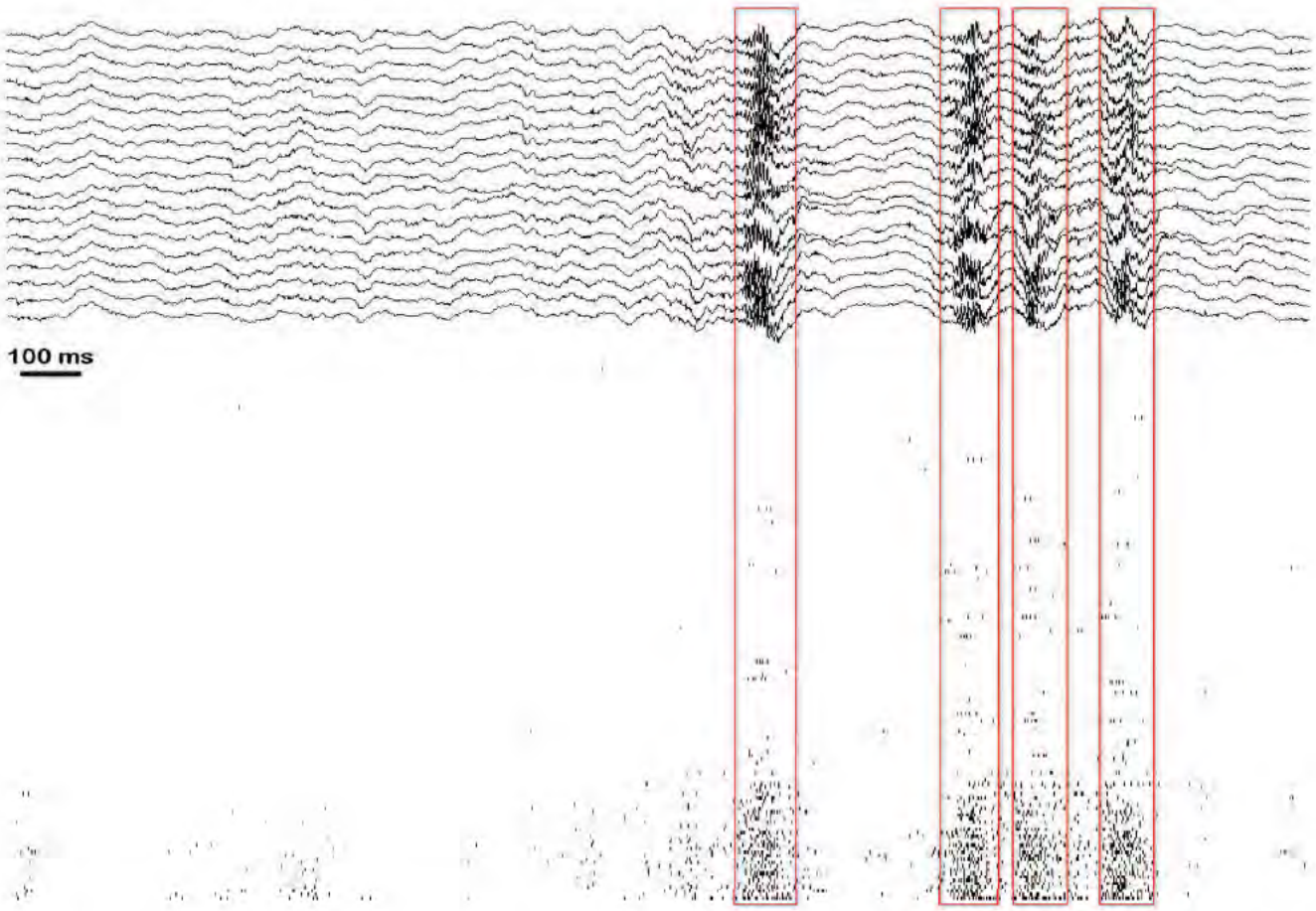

Figure 3: *Hippocampal synchronous bursts during slow-wave sleep.* Local field potential (LFP) recordings from several tetrodes in CA1 (top), together with rasters of 154 simultaneously recorded CA1 cells during slow-wave sleep. Rasters are ordered in terms of firing rates. A block of high firing interneurons is at the bottom of the figure. Sharp-wave bursts (SPWs) are marked by the red rectangles. Notice that CA1 cells tend to fire synchronously around SPWs, and that high-frequency oscillations (~200 Hz ripples) are observed in the LFPs together with sharp-wave bursts.

351. Decoupling through synchrony in recurrent networks

Evgeniy Lubenov, Thanos Siapas

Synchronization is a key property of many biological, physical, and engineering systems. The level of synchronization in distributed systems is often controlled by the strength of the interactions between individual elements. In brain circuits the connection strengths between neurons are constantly modified under the influence of spike-timing-dependent plasticity rules (STDP). However, the consequences of these local rules for the global dynamics of brain networks are not fully understood. We have shown that when recurrent networks exhibit population bursts STDP rules exert a strong decoupling force that desynchronizes activity. Conversely, when activity is random, the same plasticity rules can have a coupling and synchronizing influence. The presence of these opposing forces promotes the self-organization of spontaneously active recurrent networks to a state at the border between randomness and synchrony. These results may have implications for the transfer of information in cortico-hippocampal networks during memory formation, and for understanding the therapeutic effects of deep brain stimulation for Parkinson's disease and epilepsy.

352. Mechanisms and functional consequences of synchronous hippocampal bursts during slow-wave sleep

Evgeniy Lubenov, Casimir Wierzynski, Ming Gu, Thanos Siapas

Hippocampal activity during slow-wave sleep is characterized by the presence of highly synchronous bursts (sharp-wave bursts, Figure 3). Within each of these bursts about 40,000 CA1 cells (~10%) fire within a window of less than 100 ms. These massive population events are believed to be very effective in driving hippocampal postsynaptic targets and engaging plasticity mechanisms. We study the patterns of neuronal firing during these bursts, and analyze how these patterns evolve throughout sleep. These experimental efforts are complemented with the development of computational models of the mechanisms underlying the generation of synchronous bursts within recurrent networks.

Figure 4: *Hippocampal activity during REM sleep.* The same neurons and LFP traces as in **Figure 1** during a REM episode (2 minutes earlier than in **Figure 3**). Notice the scale change from **Figure 1**. Theta oscillations (4-10 Hz) are clearly visible in the LFPs, and interneurons fire rhythmically phase-locked to the theta oscillations. Pyramidal cells are rhythmically activated over multiple theta cycles, similar to the activation during a pass through a place field (for example the segment marked by the red rectangle).

353. Hippocampal activity patterns during REM sleep

Evgueniy Lubenov, Ming Gu, Casimir Wierzynski, Thanos Siapas

REM sleep activity looks remarkably similar to the awake activity, hence the name, *paradoxical sleep*. As illustrated in **Figure 4**, hippocampal LFPs are characterized by theta oscillations, regular 4-10 Hz oscillations that also characterize hippocampal activity during the awake behavior. Hippocampal cells fire phase-locked to the theta rhythm both in awake behavior and REM sleep, but the preferred phases in these two brain states need not be the same [1]. The timing of spikes of hippocampal neurons with respect to the theta rhythm has strong effects on synaptic plasticity [2,3] hence quantifying changes in phase-locking may provide important insights into the functional role of sleep in memory formation. We study the evolution of phase locking to theta oscillations across multiple sleep-wake cycles, and characterize the effects of experience on phase locking in sleep. In addition, we develop tools for analyzing and identifying network activity motifs in REM sleep and study their experience specificity.

References

[1] Poe, G.R., Nitz, D.A., McNaughton, B.L. and Barnes, C.A. (2000) *Brain Res.* **855**:176–180.

[2] Holscher, C., Anwyl, R. and Rowan, M.J. (1997) *J. Neurosci.* **17**(16):6470–6477.

[3] Pavlides, C., Greenstein, Y.J., Grudman, M. and Winson, J. (1988) *Brain Res.* **439**:383–387.

354. Electrical stimulation of hippocampal circuits

Evgueniy Lubenov, Thanos Siapas

We chronically implant multiple tetrodes and stimulating electrodes in order to both observe and alter neuronal activity patterns. Our efforts focus on stimulating area CA3 and its afferent/efferent pathways. Low intensity pulses produce a field EPSP (fEPSP) whose slope reflects the mean synaptic coupling in the network. We track the evolution of fEPSP slopes during the awake experience and sleep, and relate systematic changes in these slopes to patterns of CA3 population activity.

355. Prefrontal-hippocampal interactions in associative learning

Casimir Wierzynski, Thanos Siapas

Eyeblink conditioning is a form of associative learning that has been shown to engage the hippocampus across a wide range of species and parameters [1]. Moreover, in its trace form, where the conditioned and unconditioned stimuli do not overlap in time, eyeblink conditioning has been shown to require an intact hippocampus for successful acquisition [2]. This hippocampal dependence falls off with time, implying that the long-term locus of the CS-US association is extra-hippocampal. Furthermore, lesions to the medial prefrontal cortex in rats have been shown to disrupt the long-term recall of the eyeblink response, but not its acquisition [3]. Using simultaneous chronic recordings from the hippocampus and medial prefrontal cortex, we are characterizing the relationships between the activity patterns in these brain areas during the acquisition of the CS-US association. These experiments enable us to study how the CS-US association is represented across prefrontal and hippocampal circuits, and how this representation evolves with learning.

References

[1] Christian, K.M. and Thompson, R.F. (2003) *Learning & Memory* **10**:427-455.

[2] Weiss, C., Bouwmeestev, H., Power, J.M. and Disterhoft, J.F. (1999) *Behav. Brain Res.* **99**:123–132.

[3] Takehara, K., Kawahare, S. and Kirino, Y. (2003) *J. Neurosci*. **23**(30):9897-9905.

356. Dynamics of phase locking across cortico-hippocampal networks

Evgueniy Lubenov, Casimir Wierzynski, Thanos Siapas

During the awake behavior, hippocampal activity is marked by the presence of pronounced 4-10 Hz LFP oscillations known as theta oscillations [1]. We demonstrated that a significant proportion (about 40%) of the cells in the medial prefrontal cortex of the rat fire preferentially during particular phases of the hippocampal theta rhythm [2]. Furthermore, we showed that prefrontal neurons phase lock best to theta oscillations delayed by approximately 50 ms and confirmed this hippocampo-prefrontal directionality and timing at the level of correlations between single cells. Finally, phase locking of prefrontal cells is predicted by the presence of significant correlations with hippocampal cells at positive delays up to 150 ms. This indicates that direct hippocampal input is likely to have a considerable contribution to the observed prefrontal phase locking. We are studying how these phase-locking properties evolve over time and how they are modulated by behavior.

References

[1] Vanderwolf, C.H. (1969) *Electroencephalogr. Clin. Neurophys.* **26**:407–418.

[2] Siapas, A.G., Lubenov, E.V. and Wilson, M.A. (2005) *Neuron* **46**:141-151.

357. Prefrontal-hippocampal interactions during slow-wave sleep

Casimir Wierzynski, Thanos Siapas

Sleep is characterized by a structured combination of neuronal oscillations. In the hippocampus, slow-wave sleep (SWS) is marked by high-frequency network oscillations (200 Hz "ripples" - **Figure 3**), whereas neocortical SWS activity is organized into low-frequency delta (1–4 Hz) and spindle (7–14 Hz) oscillations. We have previously shown that hippocampal ripples tend to occur together with neocortical spindles [1]. This coactivation of hippocampal and neocortical pathways may be important for the process of memory consolidation, during which memories are gradually translated from short-term hippocampal to longer-term neocortical stores. We are investigating the precise patterns of firing around ripple-spindle episodes and their relationship to awake patterns.

Reference

[1] Siapas, A.G. and Wilson M.A. (1998) *Neuron* **21**:1123-1128.

358. Comparison of CA3 and CA1 place fields

Ming Gu, Thanos Siapas

Pyramidal cells ("place cells") in the hippocampal subfields CA3 and CA1 fire in specific regions of space ("place fields" – **Figure 2**). These subfields have very different intrinsic architecture: CA3 is characterized by extensive recurrent collaterals that are not prominent in CA1. Does the different architecture imply different place field properties in CA3 and CA1? We characterize the precise temporal structure of firing, and the relationships of place field firing across these subfields. We, furthermore, examine how place field representations evolve and mature when the animal is exposed to novel environments.

359. Reward learning and VTA-hippocampal interactions

Thanos Siapas

Many lines of evidence suggest that the ventral tegmental area (VTA) interacts with the hippocampus to modulate the entry of information into long-term memory [1]. The VTA-hippocampal loop has been hypothesized to be important for the detection of novelty and signaling of the behavioral relevance of stimuli in the environment. However, the organization of VTA-hippocampal interactions remains unknown, as simultaneous recordings from these areas have not been reported in the literature. Leveraging our experience with chronic multi-area tetrode recordings, we are examining the simultaneous response of multiple dopaminergic cells in the VTA and pyramidal

cells in hippocampus during exposure to novel stimuli as well as during the acquisition of instrumental conditioning paradigms.

Reference

[1] Lisman, J.E. and Grace, A.A. (2005) *Neuron* **46**:703-713.

360. Wireless recording of multi-neuronal spike trains in freely behaving animals

In collaboration with Alan Litke of the University of California, Santa Cruz, and Markus Meister of Harvard University, we are developing a wireless system that would allow recording neuronal activity from freely behaving animals. The system will acquire signals from 64 channels, amplify and filter the waveform from each channel, and transmit the data with a broadband radio transmitter to a remote receiver. This technique will enable examining neuronal network activity while animals explore much larger spatial extends than previously possible, and may enable probing neural function in natural behaviors, such as flying, that were difficult to study in the past.

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Postdoctoral Scholars: Mili Jeon, Hyung-Kook Lee, Ed Silverman
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Summary of Zinn group research: Our group studies the molecular mechanisms of axon guidance and synaptogenesis. The fruit fly *Drosophila melanogaster* is our primary experimental system. In the embryo, we examine signaling systems involved in motor and CNS axon guidance. In the larva, we study synaptogenesis and synaptic plasticity in the neuromuscular system. Our approaches combine genetics, molecular biology, electrophysiology, biochemistry, and cell biology. The current group members include four postdocs (Kaushiki Menon, Mili Jeon, Ed Silverman, and Peter (Hyung-Kook) Lee, two graduate students (Anna Salazar and Ashley Wright), an Assistant Biologist (Lakshmi Bugga), a lab manager/technician (Violana Nesterova), a technician working on motor axon targeting (Amy Cording), and a technician participating in various projects by performing dissections and *Drosophila* crosses (Elena Armand). Abstracts included here provide information about P. Lee's, E. Silverman's and L. Bugga's work. The rest of the work in the lab is described in this Summary.

Motor axon guidance and muscle targeting

The *Drosophila* motor axon network has provided one of the best systems in which to study axonal pathfinding mechanisms. The network is simple: 32 motoneurons innervate 30 body wall muscle fibers in each abdominal segment. Each motoneuron axon is targeted to a specific muscle fiber, and very few projection errors are made during normal development. Thus, the motor axon network is a genetically hard-wired map, and is an ideal system in which to study how genes control the formation of specific synaptic connections. In much of our work, we have focused on the roles of tyrosine phosphorylation in regulating motor axon guidance decisions. We are now conducting genetic screens to determine the mechanisms by which cell surface proteins label specific muscle fibers for recognition by motor axon growth cones.

Targeting of motor axons to specific muscle fibers

Despite the advances in characterizing molecules that regulate motor axon pathfinding, we still understand little about how specific muscle fibers are recognized as targets for synapse formation by these axons. Many mutations affect pathfinding decisions, leading to aberrant wiring of the neuromuscular system, but no single loss-of-function (LOF) mutations are known that block recognition of specific muscle targets. These results are most easily explained by invoking genetic redundancy in target labeling. If each muscle fiber were defined by a combination of several cell surface labels, removing one of the labels might not have a major effect on targeting of axons to that fiber. This would explain why targeting molecules have not been identified in conventional LOF genetic screens.

Studies of gain-of-function (GOF) phenotypes by other groups are consistent with the redundancy hypothesis. For example, the homophilic cell adhesion molecule Fasciclin III (Fas III) is expressed on only two muscle fibers, 6 and 7, and on the growth cone of the RP3 neuron that innervates these two fibers. Fas III appears to be a functional target label, because when it is ectopically expressed on other muscle fibers near 6 and 7, the RP3 neuron makes abnormal synapses on these Fas III-expressing fibers. However, when Fas III is removed by a LOF mutation, there is no effect on targeting of RP3 to 6 and 7. These results imply that Fas III can be used for muscle targeting, but that targeting of 6 and 7 can still proceed in its absence, presumably because these fibers are also labeled by other surface molecules that can be recognized by the RP3 growth cone when Fas III is not present.

These findings suggest that cell-surface proteins that label specific targets in the motor axon system might be identifiable by a GOF genetic screen in which candidate labels are ectopically expressed on all muscle fibers. If these proteins are functional labels, their misexpression might produce alterations in target recognition, as observed in the Fas III experiments described above. By identifying genes encoded in the *Drosophila* genome that can confer GOF phenotypes in which targeting of specific muscle fibers is altered, we will acquire the tools to understand the mechanisms involved in target recognition in this system. This type of screen should allow us to overcome the redundancy problem. For example, suppose one could identify three different cell-surface proteins that are normally expressed on a specific muscle fiber, but whose misexpression on other muscle fibers produces targeting errors. One might then predict that removing all three of these proteins by making a triple LOF mutant (through conventional or RNAi techniques) would now prevent targeting of this muscle fiber. Through these kinds of experiments, we could begin to understand the combinatorial code for muscle targeting. Insights into the motor axon targeting code would be likely to facilitate an understanding of targeting in other neuronal systems (e.g., the antennal lobe, optic lobe, and mushroom body), since candidate target labels are usually expressed by a variety of neuronal and non-neuronal cell types.

To conduct this GOF screen, we first created a database of all cell-surface and secreted (CSS) proteins in *Drosophila* that are likely to be involved in specific cell-cell interactions. The database was generated by database mining and reiterative computational screening. We

defined all fly genes encoding proteins that contain domains known to be present in CSS proteins in other eukaryotes (including all of the 240 domains in the 'extracellular' portion of the SMART database, http://smart.embl-heidelberg.de/browse.shtml, that are represented in flies). We then eliminated several hundred genes that we thought were unlikely to be important for cell recognition, and defined a CSS cell recognition candidate collection of about 1005 genes.

To drive expression of these genes in muscles, we used the 'EP' system, in which a P element containing a block of UAS sequences that are responsive to the yeast transcription factor GAL4 is jumped around the genome. Like other P elements, EPs usually land upstream of genes. If a line bearing an EP upstream of a gene is crossed to a 'driver' line expressing GAL4 in all muscle fibers, the gene will now be expressed at high levels in muscles in the resulting progeny embryos and larvae. To find EP-like elements upstream of the CSS genes, we searched through about 40,000 different insertions that have been maintained in collections of *Drosophila* lines. These include the original EP set generated by Pernille Rorth, the EY insertion lines generated in the Bellen lab, the GS lines developed in Japan, insertions generated by Exelixis, Inc. and maintained at Harvard, and the GE lines developed by GenExel, Inc. We were able to identify insertions that can confer expression of 421 of the 1005 CSS genes in our database, representing about 40% of the repertoire and including members of all CSS protein families.

To screen for genes encoding potential targeting molecules, we crossed each of these insertions to a strong pan-muscle GAL4 driver and visualized motor axons and neuromuscular junction synapses in the resulting F1 progeny larvae by immunostaining. We have already identified over 30 genes that cause synaptic mistargeting on muscles 12 and 13, and ~160 genes that cause synaptic morphology phenotypes.

We have focused initially on the analysis of the mistargeting genes, as this is our primary interest and there are fewer of these to consider. We began by confirming the phenotypes with other GAL4 drivers. We then evaluated the normal expression patterns of the mRNAs encoding these genes, using *in situ* hybridization and antibody staining. This showed that many of the mistargeting genes are normally expressed in muscles or in regions of the periphery that are contacted by motor axons during axon outgrowth. We then studied LOF phenotypes for the genes by obtaining insertion mutations in or near the genes that reduce their expression, and by knocking down expression using transgenic RNAi techniques. We have already identified at least six genes for which reducing expression produces embryonic or larval phenotypes. The data thus far suggests that one gene encodes an epidermal protein that is necessary to drive motor axons away from the epidermis and toward their muscle targets. Another gene encodes a neural receptor necessary for ISNb guidance. Four genes may encode the type of proteins we were searching for in the screen: cell surface proteins that selectively regulate targeting of motor axons to specific muscle fibers.

We are continuing to examine these and other genes identified by the screen. Our long-term goal is to establish how the members of the gene families represented in the mistargeting gene collection work together to confer an accurate pattern of motor axon targeting. This will require making double and multiple mutants (or RNAi knockdowns that affect more than one protein expressed on an individual muscle fiber (M. Kurusu, A. Cording *et al*., manuscript in preparation).

Neural receptor tyrosine phosphatases

In the 1990s, we showed that receptor-linked protein tyrosine phosphatases (RPTPs) are selectively expressed on CNS axons and growth cones in the *Drosophila* embryo, and that these RPTPs regulate motor and CNS axon guidance during embryonic development. RPTPs directly couple cell recognition *via* their extracellular domains to control of tyrosine phosphorylation *via* their cytoplasmic enzymatic domains. The extracellular regions of the fly RPTPs all contain immunoglobulin-like (Ig) and/or fibronectin-type III (FN3) domains, which are usually involved in recognition of cell-surface or extracellular matrix ligands. Their cytoplasmic regions contain either one or two PTP enzymatic domains. The fly genome encodes six RPTPs (LAR, PTP10D, PTP69D, PTP99A, PTP52F, PTP4E), and we have generated or obtained mutations in all six of the genes encoding these proteins.

We have now performed a detailed characterization of the genetic interactions among all six RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs; thus, in a sense, there is an "RPTP code" for each decision. In some cases, the RPTPs work together, so that defects are only observed when two or more are removed. In other cases, however, phenotypes produced by removal of one RPTP are suppressed when a second RPTP is also absent. Our results provide evidence for three types of relationships among the RPTPs: partial redundancy; collaboration; and competition. Our most recent work will be described in a manuscript to be submitted soon that analyzes the complete pairwise genetic interaction matrices for control of CNS longitudinal tract and motor axon guidance mediated by all six of the RPTPs (M. Jeon *et al.*, manuscript in preparation). Our major efforts in this area are now directed toward understanding these relationships at the biochemical level, through definition of upstream (ligands) and downstream (substrates) components of RPTP signaling pathways (see below).

A genetic approach to identification of RPTP ligands

The ligands recognized by RPTPs *in vivo* have not been identified in any system. In order to understand how RPTPs regulate axon guidance, it is essential to know when and where they engage ligands, and how ligand binding affects enzymatic activity and/or localization.

One of our current approaches to identifying ligands is based on our observation that fusion proteins in which the extracellular domains of RPTPs are joined to human placental alkaline phosphatase (AP) can be used to stain live-dissected *Drosophila* embryos. Each of four fusion proteins (LAR-AP, PTP69D-AP, PTP10D-AP, PTP99A-AP) binds in a specific manner. Each fusion protein stains a subset of CNS axons and also binds to other cell types in the periphery. To identify the genes encoding the RPTP ligands, we are screening deficiency mutations that remove specific portions of the genome. We began by screening the Bloomington 'deficiency (Df) kit' of 266 fly lines. Each Df line was crossed to GFP balancers so that Df/Df embryos could be identified, and we then stained these embryos with each of four fusion proteins (LAR-HS2-AP, 69D-AP, 10D-AP, 99A-AP). Since each Df lacks a specific region of the genome, if homozygous Df embryos do not stain with a fusion protein, this indicates that this genomic region contains a gene required for ligand expression. Overlapping Dfs and point mutants can then be screened in order to identify the relevant gene within the Df.

Using this screen, we found a Df that contains a gene encoding a ligand that binds to LAR-AP, and have identified this ligand as Syndecan (Sdc). This work has been published [Fox, A.N. and Zinn, K. (2005) *Curr. Biol.* **15**:1701-1711]. The heparan sulfate proteoglycan Syndecan is an *in vivo* ligand for the *Drosophila* LAR receptor tyrosine phosphatase. Sdc is a heparan sulfate proteoglycan (HSPG). Our results show that LAR binds to the glycosaminoglycan side chains of Sdc with nanomolar affinity, and that Sdc is required for DLAR-mediated axon guidance. We can generate motor axon guidance errors by overexpressing LAR on neurons, and find that the same errors are generated by ectopically expressing Sdc on muscles. This Sdc GOF phenotype is suppressed by LOF mutations in the *Lar* gene, indicating that LAR is epistatic to (downstream of) Sdc. This result shows that muscle Sdc can function in *trans* as a ligand for LAR on neuronal growth cones, and suggests that binding to Sdc increases LAR's signaling activity.

We have continued the Df screen, and have identified four regions required for 99A-AP staining. Ashley Wright is now screening overlapping Dfs and point mutations to find the responsible genes. Our results thus far already indicate that a novel glial-neuronal interaction is required to specify expression of the 99A ligand.

Our approach is general, and can be used to identify ligands for any 'orphan receptor' that has a *Drosophila* ortholog. We also used the method to define genomic regions required for expression of selected cell surface antigens, including those recognized by the 1D4 and BP102 monoclonal antibodies (mAbs). As part of the analysis, we have defined a new Df kit for embryonic screening, which uses alternative Bloomington Dfs to allow screening of regions of the genome whose removal in the normal Df kit causes early developmental failure. This new kit contains about 450 lines, and covers about 89% of polytene chromosome bands. It can be used to analyze any region of the genome for the desired embryonic phenotype. We have already analyzed about half of the genome for regions necessary for motor axon guidance by staining Df embryos with 1D4 (A.N. Fox, A. Wright *et al*., manuscript in preparation).

A gain-of-function screen for RPTP ligands

Despite the success of the Df screen (an LOF approach), it is clearly not capable of identification of all RPTP ligands, and may not even be capable of finding most of them. First, about 11% of the genome still cannot be screened, either because no Dfs exist there or because embryos homozygous for those regions do not develop. Second, and most important, the four RPTP-AP probes all stain subsets of CNS axons in addition to other patterns outside the CNS. If multiple ligands for an RPTP were all expressed on CNS axons, removal of one ligand gene by a Df might not perturb staining enough to detect a difference from wild type. We already know that this is the case for LAR: Sdc is expressed both on CNS axons and in the periphery, but only peripheral staining is eliminated in an *Sdc* mutant. CNS axons in *Sdc* mutants continue to stain with LAR-AP, and are also stained by a mutant version of LAR-AP that cannot bind to Sdc[7]. These data show that there is at least one non-HSPG ligand for LAR that is expressed on CNS axons together with the HSPG ligand Sdc. Because of these limitations, we have developed a new GOF approach to ligand screening that allows direct identification of proteins that bind in embryos to an RPTP probe, regardless of whether such proteins are normally expressed in patterns that overlap with those of other ligands. This approach is also general and can be applied to any orphan receptor of interest that has *Drosophila* orthologs. It is based on observations made by [Fox, A.N. and Zinn, K. (2005) *Curr. Biol.* **15**:1701-1711] who showed that when Sdc is ectopically expressed on muscle fibers, this produces ectopic muscle staining with LAR-AP, which normally does not bind to muscles. Thus, if one were able to express ligand genes in new patterns in the embryo, one would expect to be able to see additional staining with RPTP-AP probes and identify ligands in this manner.

Our approach is a directed EP screen. It uses the collection of EP element lines described above to ectopically express CSS proteins in new patterns in the embryo. To screen for new RPTP ligands, we are crossing each line in our CSS EP collection to GAL4 driver lines that confer ectopic gene expression in cells that normally do not stain with RPTP-AP fusion proteins. If I detect new staining patterns in embryos derived from such a cross, this may indicate that the gene driven by that EP-like element encodes a protein that can bind to the RPTP. We

have already found a number of such lines, as described below. This screen is ongoing (see Lee abstract).

Searching for RPTP substrates

It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity *in vitro*. To find substrate candidates, we performed yeast two-hybrid screens with 'substrate-trap' mutant versions of PTP10D, PTP69D, PTP52F, and PTP99A. These 'trap' proteins form stable complexes with tyrosine-phosphorylated substrates because they bind normally but cannot catalyze dephosphorylation. We introduced a constitutively activated chicken Src tyrosine kinase into yeast together with the PTP trap constructs and the cDNA library, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. We identified several classes of clones whose interactions with the substrate-trap RPTPs are dependent on coexpression of the tyrosine kinase, suggesting that they may be substrates. These include a cell-surface receptor identified with PTP52F, as well as some intracellular signaling proteins. For the receptor, we expressed a GFP-tagged version in transfected *Drosophila* S2 cells, and showed that this tagged protein selectively interacts with a cotransfected tagged substrate-trap PTP52F construct. Importantly, association in the transfected cell system requires simultaneous coexpression of the Src tyrosine kinase, and association is not observed if an enzymatically active version of PTP52F is used instead of the trap mutant. Both of these characteristics imply that the receptor is indeed a genuine PTP52F substrate. We are currently mapping the tyrosine(s) that are substrates for PTP52F dephosphorylation using this trap cotransfection assay (Bugga abstract).

Tracheal development: Localization of apical proteins to the tracheal lumen is controlled by RPTPs

In the process of examination of double mutants lacking expression of the closely related proteins PTP4E and PTP10D, we noticed that the tracheal network exhibits severe defects in these embryos. We have shown that these defects, which include formation of huge 'bubbles' along the tracheal tubes, arise from mislocalization of apical proteins to intracellular vesicle compartments. Localization of basolateral proteins is unaffected. This mislocalization phenotype also involves dysregulation of Rho GTPases and receptor tyrosine kinases. Our current work is directed toward a mechanistic understanding of the relationships among the proteins whose perturbation gives rise to these apical targeting phenotypes (M. Jeon and K.Z., manuscript in preparation).

Genes controlling synaptogenesis in the larval neuromuscular system

Motor growth cones reach their muscle targets during late embryogenesis and then mature into presynaptic terminals that are functional by the time of hatching. The pattern of Type I neuromuscular junction (NMJ) synapses in the larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber. These synapses continue to expand and change as the larva grows, because their strengths must be matched to the sizes of the muscle fibers they drive. This growth represents a form of synaptic plasticity, because it is controlled by feedback from the muscle to the neuron. Studies of NMJ synapses in flies are relevant to an understanding of synaptic plasticity in the mammalian brain, because the fly NMJ is a glutamatergic synapse, organized into boutons, that uses ionotropic glutamate receptors homologous to vertebrate AMPA receptors.

Control of synaptic local translation by Pumilio and Nanos

Our recent work on synapses has focused on control of synaptic protein translation. Local translation at synapses has been studied in *Aplysia*, mammalian, and arthropod systems. It has attracted interest because it is a mechanism that allows neurons to separately adjust the strengths of individual synapses.

To identify genes involved in synaptogenesis in larvae, including those that regulate local translation, we devised and executed a GOF screen of live third instar larvae. In the screen, we identified *pumilio* (*pum*), which encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. Translational repression by Pum controls posterior patterning during embryonic development. In a 2004 paper, we showed that Pum is an important mediator of synaptic growth and plasticity at the NMJ. Pum is localized to the postsynaptic side of the NMJ in third instar larvae, and is also expressed in larval neurons. Neuronal Pum regulates synaptic growth. In its absence, NMJ boutons are larger and fewer in number, while Pum overexpression increases bouton number and decreases bouton size. Postsynaptic Pum negatively regulates expression of the essential translation factor eIF-4E (the cap-binding protein) at the NMJ, and Pum binds selectively to the 3'UTR of *eIF-4E* mRNA. These data suggest that Pum is a direct regulator of local eIF-4E translation, and that eIF-4E (which is normally limiting for translation) in turn switches on translation of other synaptic mRNAs. Pum also directly regulates the GluRIIa glutamate receptor. These results, together with genetic epistasis studies, suggest that postsynaptic Pum modulates synaptic function *via* direct control of local synaptic translation.

In our current work, we have identified the Pum cofactor Nanos, which works together with Pum to repress translation in the early embryo, as a participant in Pum regulation of targets at the NMJ. In *nos* mutants (or transgenic *nos* RNAi larvae), GluRIIa is upregulated as in *pum* mutants, and there are changes in presynaptic terminal morphology at the NMJ. Interestingly, eIF-4E is not strongly upregulated in *nos* mutants, consistent with the observation that Pum-eIF-4E mRNA complexes are not able to recruit Nos. Pum also regulates Nos levels at the NMJ; this may be an important autoregulatory control mechanism (K. Menon *et al*., manuscript in preparation).

Assembly of Pumilio into ordered aggregates as a regulatory switching mechanism

We are also studying Pum in another context: its potential role as a prion-like switch that could control synaptic translation *via* regulated assembly into an ordered aggregate. This project emerged from a computational search we performed to identify switch proteins that might have the capacity to form ordered aggregates. This is relevant to human disease, as well, since proteins involved in many human neurodegenerative diseases share a propensity to form amyloid aggregates. One class of sequences that can form amyloids are domains rich in glutamine (Q) and asparagine (N). These are present in many metazoan proteins, including ~450 in *Drosophila*. Q/N domains are found in all yeast prions, and these domains have been positively selected during evolution, perhaps in order to allow reversible switching of the functional domain of the prion into an inactive aggregated state. We wondered this type of selection might also maintain Q/N domains in metazoans. To examine this question, we devised a computational search strategy to identify candidates for nucleic-acid binding prion switches in metazoan proteomes.

One of the two strong *Drosophila* candidates identified in this search is Pum. As described above, work by our group had shown that Pum is localized to the postsynaptic side of the larval NMJ, where it acts as a regulator of local mRNA translation. We found that a Q/N-rich domain (denoted NQ1) from Pum exhibits prion-like behavior in budding yeast, including heritable phenotypic switching and reversibility by guanidine hydrochloride. NQ1 purified from *E. coli* converts *in vitro* to an aggregated form that exhibits amyloid-like characteristics, including formation of fibers and Congo Red birefringence. To test whether NQ1 aggregate formation can perturb Pum's function in the nervous system, we created transgenic fly lines in which NQ1 expression is driven by GAL4. Our results show that postsynaptic NQ1 expression generates alterations in the NMJ that phenocopy the *pum* loss-of-function phenotype and interact genetically with *pum* mutations. Postsynaptic Pum overexpression is lethal, but co-overexpression of NQ1 rescues this lethality, suggesting that NQ1 can inactivate endogenous Pum. We are currently investigating whether amyloid formation is a pathological state or a normal regulator of Pum activity *in vivo* (A. Salazar, E. Silverman, manuscript in preparation; see Silverman abstract).

361. Identification of RPTP substrates

Lakshmi Bugga

To isolate RPTPs in a complex with their target substrates, we are using yeast two-hybrid system. We are looking for proteins that interact with *Drosophila* neuronal RPTPs-10D, 69D, 99A and 52F. To attempt to achieve stable binding of the RPTPs to a tyrosine phosphorylated substrate, we have used 'substrate-trap' mutants of the RPTPs, which can bind to substrates but do not catalyze dephosphorylation, instead remaining bound to substrate in a stable complex. We constructed plasmids encoding GAL4 DBD/RPTP bait proteins and introduced them into yeast together with fly cDNA libraries encoding GAL4AD-cDNA fusion proteins. We also introduced a plasmid containing a constitutively active form of chicken c-Src, driven by a constitutive yeast promoter. Positive interactions are detected by selecting on plates lacking the auxotrophic marker and screening for reporter expression.

Our screen with all four RPTPs resulted in several positive clones -about 15 genes that interact with any of the four RPTPs. We identified seven genes that interact specifically with a given RPTP, four of these genes as potential substrates based on Src dependence. The most promising potential substrates is the cell-surface receptor Tartan. We are currently testing Tartan interactions *in vitro* by transient transfection experiments with *Drosophila* cell line-S2 cells and finding out the expression of rest of the unknown genes by RNA *in situs*, and looking for double mutant phenotypes with RPTP and tartan mutant flies. Preliminary results with tartan mutants showed branching defects in SNa, and similar defects between 52F and tartan mutants (A. Ratnaparkhi, unpublished).

Our biochemical experiments with full-length *tartan* showed association with substrate-trap 52f, and this association decreased/disappeared with the wild type 52F, showing that tartan is a substrate for 52f. In our GST pull down experiments, when 52f trap fusion protein was used to pull down Tartan cytoplasmic domain protein, 52trap fusion protein precipitated endogenous tartan more efficiently than the truncated form. We are currently investigating to see if pull downs with tartan FL would behave same way. We are also trying to map the area of the tartan gene that specifically interacts with 52F. We made five different genes to delete several different combinations of the 11 tyrosine residues present in the cytoplasmic domain. Our preliminary experiments show that most of these tyrosines are getting phosphorylated. This could be because we are removing more than one tyrosine in each gene, or it could be that these proteins are running close to the heavy chain, and difficult to predict the extent of phosphorylation. We are currently making deletions in the cytoplasmic domain of the full-length gene to check phosphorylation.

362. Identification of receptor protein tyrosine phosphatase ligands in *Drosophila*

Hyung-Kook Lee

The goals of my study are to identify the ligands of RPTPs and to elucidate the mechanisms of axon guidance through RPTP signaling using the *Drosophila* system. This study adopts a gain-of-function (GOF) genetic screen to find RPTP ligands that affect axon guidance. We selected about 300 genes from a database of all cell-surface and secreted proteins in *Drosophila*, which possibly involved in specific cell-cell interactions. The expression of the selected genes in developing embryos has been conducted by using the 'EP' system and GAL4 drivers. Screening of RPTP ligands in *Drosophila* system has been performed by using the fusion proteins, which the extracellular domains of RPTPs (PTP10D, PTP69D,

PTP99A and PTPHS2) jointed to human placental alkaline phosphatase (AP). These fusion proteins have been used to stain live *Drosophila* embryos that expressing candidate labels ectopically.

By GOF screen, I found that *Stranded at second* (*Sas*), a putative epidermal cell surface receptor that required for larval development, is a strong candidate for DPTP10D. When *Sas* was over-expressed in embryos during development, the ectopic muscle surface staining of DPTP10D-AP has been observed. This staining pattern appears identical to the muscular system. Possibly, the FN3 domains of the *Sas* protein on the surface of muscles bind to the extracellular region of DPTP10D-AP fusion protein.

363. The *in vitro* aggregation of an NQ-rich region of *pumilio*

E. Silverman

We are interested in whether the NQ-rich region of pumilio has the capability to aggregate and, if so, whether this aggregation can alter the function of *pumilio*. Anna Salazar in our lab has shown that a Q/N rich region of Pumilio can aggregate in yeast and studies are currently under way investigating potential *in vivo* effects in flies. Another way we are addressing this is by analyzing the NQ-rich region of pumilio *in vitro*. Using a canonical NQ-rich region of yeast protein Sup35 known to aggregate in a prion-like manner, NM, as a positive control, and the highly charged M region of Sup35 as a negative control, we purified the first NQ-rich region of pum (NQ1) and NQ1 lacking glutamine-rich stretches (NQ-Q) and allowed these proteins to spontaneously convert with mild agitation into an aggregated state.

First, we looked at solubility in 2% SDS. A protein capable of forming amyloid will be insoluble in SDS without boiling. After conversion, NQ1 and NM are insoluble in SDS without boiling, whereas M remains soluble and NQ-Q is mostly soluble. This result indicates that NQ1 is capable of forming amyloid. NQ1 and NM fibers birefringe under cross-polarized light when stained with Congo Red, which is used to diagnose amyloid in disease and *in vitro*. Fibers of NM and NQ1 are recognized by negatively stained TEM. When a highly conserved, charged region of NQ1 (CT) is removed from NQ1, the conversion to the SDS-insoluble state is more complete and appears to occur more readily. When this region is expressed alone, it fails to change into an SDS-insoluble state, indicating that it may have a role in preventing non-specific aggregation of Pum.

We plan to test a yeast strain carrying a chimeric gene where NQ1 replaces the NM region of Sup35 for the ability of the *in vitro* fibers to be infectious. We will also test other regions of Pumilio and accessory domains (non NQ) in NQ1 to better understand the conversion and aggregation events *in vivo*.

Publications

Kurusu, M. and Zinn, K. (2007) Receptor tyrosine phosphatases regulate birth order-dependent axonal fasciculation and midline repulsion during development of the *Drosophila* mushroom body. Manuscript submitted for publication.

Ratnarparkhi, A. and Zinn, K. (2007) The secreted cell signal Folded Gastrulation regulates glial morphogenesis and axon guidance in *Drosophila*. *Dev. Biol.* **308**:158-168.

Zinn, K. (2007) Dscam and neuronal uniqueness (Review). *Cell* **129**:455-456.

Structural, Molecular and Cell Biology

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Summary: The work of our laboratory in recent years has focused on the origins of replication in mitochondrial DNA (mtDNA) and how they are differentially regulated, as well as on aging and longevity mutations, their detection and their effect on mitochondrial function. A few years ago, we had identified a new major origin of heavy-strand replication at position 57 in the human mtDNA control region (1). This origin is the major one responsible for the replication of mtDNA under normal maintenance conditions. Under conditions in which mtDNA must be rapidly synthesized to re-establish its normal level in the cell, the classical heavy-strand origins, clustered between positions 191 and 151, are called into play. According to the model of asymmetric mtDNA replication, replication of the light strand begins after more than 70% of the heavy strand has been replicated. Replication of this strand starts at O_L between positions 5721 and 5798 (2). In the past year, we have searched for additional origins of replication, and have found two new light-strand origins, located in the control region. How these are used in conjunction with O_L remains to be elucidated.

Our interest in replication has prompted us to investigate the structure of mitochondrial nucleoids, the DNA/protein/membrane complexes in which the mitochondrial genome is organized within the mitochondrion. Using the double-stranded DNA-specific stain PicoGreen, we were able to study the nucleoid structure and abundance in mutant cells. Of striking interest was the effect of mutations in complex I on nucleoid configuration.

We have continued the analysis of the phenotypes associated with the T414G fibroblast-specific aging mutation. This mutation occurs in the control region of mtDNA, specifically, near the light strand promoter, which is used to make primers for DNA synthesis of the heavy strand. We have used for our analysis cybrid clones carrying 100% wild-type mtDNA or 100% mutant mtDNA. These were derived from heteroplasmic fibroblast strains from three individuals by hybridization of cytoplasts with mtDNA-less cells. These clones have the same nuclear background, i.e., that of 143B ρ^o zero cells. Thus, the phenotypic differences among the clones are presumably due only to mtDNA variation. We have found no consistent trend of change in biochemical phenotype or in DNA replication levels which could be related to the presence of the T414G mutation: thus, this mutation appears to be neutral. On the other hand, the presence of the C150T longevity mutation, as studied in two haplogroup-matched pairs of individuals, appeared to correlate with lower reactive oxygen species (ROS) production levels.

We take this opportunity to reflect on the many advances that have been made in the field of mitochondrial biogenesis and genetics over the course of the 40 years during which we have been working in these areas. Indeed, the picture that we have today on mtDNA and its products is far richer and more detailed than the bare sketches that we had in 1969. This is due to the work of many young colleagues with whom we have had the pleasure to collaborate and to the contributions of excellent laboratories around the world. We are humbled and excited to see that there are still many unsolved mysteries in these areas of mitochondrial research.

References

(1) Fish, J., Raule, N. and Attardi, G. (2004) *Science* **306**:2098-2101.
(2) Tapper, D.P. and Clayton, D.A. (1981) *J. Biol. Chem.* **256**:5109-5115.

364. Alternative origins for mitochondrial DNA L-strand replication

Nahoko Iwata, Nicola Raule, Anne Chomyn

According to the asymmetric model of mitochondrial DNA (mtDNA) replication, synthesis of the heavy (H-)strand is initiated first, at one of several sites in the control region. After a major part of the H-strand has been synthesized, synthesis of the L-strand begins. The origin of light- (L-)strand replication occurs near position 5700 (1), approximately 67% of the way around the genome from the H-strand origins. Origins of replication corresponding to this model can be identified by mapping the 5' ends of nascent DNA chains. A few years ago, Fish *et al.* (2) identified a previously undiscovered H-strand origin at nucleotide position 57, downstream of the cluster of classical H-strand origins. This origin was found to be the major origin responsible for the replication of mtDNA replication under maintenance conditions. This finding of a new origin prompted us to search the H- and L-strands for additional origins of replication.

To detect nascent DNA chains starting from unidirectional origins and to map their 5' ends, we applied the approach used by Zhang *et al.* (3) which involves annealing a 5'-end-labeled synthetic oligonucleotide to denatured DNA and extending the primer with DNA polymerase until the 5' end of the template is reached (3). The size of the extended primer, and therefore the position of the 5' end, is ascertained by gel electrophoresis. We probed for nascent chains of both strands around the entire genome by annealing and extension of primers spaced at intervals of 3000 nucleotides or less.

We initially detected many primer extension products, most of which were of low abundance. We have refined the conditions of primer extension and have determined that most of these primer extension products were artifactual. However, we have been able to identify two new 5' ends on the L strand, presumably corresponding to origins of replication, previously undiscovered.

References

(1) Martens, P.A. and Clayton, D.A. (1979) *J. Mol. Biol.* **135**:327-351.
(2) Fish, J., Raule, N. and Attardi, G. (2004) *Science* **306**:2098-2101.
(3) Zhang, J., Asin-Cayuela, J., Fish, J., Michikawa, Y., Bonafé, M., Oliveri, O., Passarino, G., De Benedictis, G., Franceschi, C. and Attardi, G. (2003) *Proc. Natl. Acad. Sci. USA* **100**:1116-1121.

365. Respiratory Complex I activity affects the mode of mtDNA replication and the shape of nucleoids in human mitochondria

Jaehyoung Cho, Heenam Park

The mammalian respiratory Complex I (NADH-ubiquinone oxidoreductase) is one of the largest known enzymes, consisting of 46 subunits in bovine heart (1). Its enzymatic activities and its functions in mitochondrial metabolism are not understood completely. Mammalian mtDNA encodes seven subunits of Complex I. Some mutations in the coding regions have been reported as causal factors of Leber's hereditary optic neuropathy (LHON) (2). In addition, a growing number of reports has suggested that defects of Complex I assembly and of its activity can play a major role in degenerative disorders in the human neuro-muscular system, making Complex I one of the major targets of therapeutic study of human neuromuscular disorders (3).

To understand the effects of complex I deficiency on mitochondrial biogenesis, we tested mtDNA metabolism in human cellular model systems containing a point mutation in one of the mtDNA genes encoding subunits of Complex I. We have used the C4T and C9T cell lines carrying an ND4 and an ND5 mutation, respectively (4). We examined the mode of mtDNA replication in the cell lines with Complex I deficiency. The usage of mtDNA replication origins in the mutant cells showed interesting differences from that in 143B cells, which carry wild-type mtDNA. The usage of the conventional origins, those at positions 110, 151 and 191, was dramatically decreased and, in contrast, the origin at position 57 was overused. The mtDNA content in the similar tested cells was, as determined from fluorescent microscopic images of cells stained with the quantitative double-stranded DNA-specific dye, PicoGreen (5). Very interestingly, we found shape differences of the mt-nucleoid between the 143B and the C4T and C9T cells. In contrast to the well-defined round shape of mt-nucleoid in the wild-type cell (143B), the mutant cells have randomly spaced and extended nucleoid structures. The distances between two mt-nucleoids are very consistent in the wild-type cells, but are totally random in the mutant cells. To test whether these defects of mtDNA metabolism in the mutant cells result from the defect of enzymatic function of Complex I and the respiratory chain, we used a C4T mutant cell line derivative that overexpresses yeast Ndi1 protein, which is a single-subunit NADH dehydrogenase (4). In the complemented cells (C4TAAV), the respiratory function was restored to a normal level (4) and the usage patterns of replication origins also were restored. Therefore, the very first step in determining the mode of mtDNA replication seems to be affected by the status of Complex I enzymatic activity and NAD+/NADH or ADP/ATP ratio in the mitochondrial matrix. We are now quantifying both ratios in mutant and wild-type cells. More interestingly, we found the mt-nucleoid shape of C4TAAV cells was not restored to a globular structure. It seems that the morphology of the mt-nucleoid may be related to the protein structure of Complex I rather than its enzymatic activity. The defects in the mode of replication and the shapes of mtDNA nucleoid in the mutant cells may influence mtDNA biogenesis, especially mtDNA segregation.

In conclusion, this study suggests that Complex I, has in addition to its role in respiration, important functions for mitochondrial biogenesis that have not yet been visited, and which may provide meaningful clues to understand the broad and complicated phenotypes of human disorders caused by Complex I deficiency.

References

(1) Carroll, J., Fearnley, I.M., Shannon, R.J., Hirst, J. and Walker, J.E. (2003) *Mol. Cell Proteo.* **2**(2):117-126.
(2) Brown, M.D. and Wallace, D.C. (1994) *J. Bioenerg. Biomemb.* **26**:273-289.
(3) Lenaz, G., Baracca, A., Fato, R., Genova, M.L. and Solaini, G. (2006) *Ital. J. Biochem.* **55**(3-4):232-253.
(4) Bai, Y., Hajek, P., Chomyn, A., Chan, E., Seo, B.B., Matsuno-Yagi, A., Yagi, T. and Attardi, G. (2001) *J. Biol. Chem.* **276**(42):38808-38813.
(5) Ashley, N., Harris, D. and Poulton, J. (2005) *Exp. Cell Res.* **303**(2):432-446.

366. Different phenotypes derived from the aging-dependent T414G mutation in human mtDNA are possibly associated with co-segregating mutations

Paola Sgobbo, Anne Chomyn

The T at position 414 in the control region of mtDNA is mutated to G in the fibroblasts of about 50% of individuals over the age of 65. This mutation is found in heteroplasmic form (present together with wild-type mtDNA) and has been observed to be present in different proportion in first and second fibroblast samples taken from the same individual. Both of these observations indicate the somatic nature of this mutation (1). Since its position is at a critical control site for mtDNA replication, it is possible that its presence influences either mtDNA replication and/or some biochemical parameters of

mitochondria. In order to investigate the role that the mutation could play and the reason for its presence in old subjects, our studies were focused especially on a possible association of the mutation with a particular phenotype. In particular, we studied some biochemical aspects of transformant cells in which the nuclear background is that of mtDNA-less 143B ρ^o cells, while the mtDNA is that of mutation-carrying fibroblasts. Three different donors of fibroblasts were used to form transformant cell lines, among which we chose lines carrying mitochondria with mtDNA either 100% mutant or 100% wild type for the T414G mutation. Measurement of the rate of respiration, either endogenous or substrate-driven, showed in the cybrids called Ac higher values in the mutant cells than in the control cells, for the cybrids called E higher values for one mutant and one control cell line and lower values for another mutant and another control line while for the third set of cybrids called L, the values were similar to one other. These results seemed to suggest that, despite our first hypothesis, there was no correlation between the respiratory capacity of mitochondria and the presence of the T414G mutation. Experiments were then carried out to verify if the mutation could lead to a higher mtDNA replication rate. For this purpose, Quantitative Real Time PCR was carried out but the results, once again, did not seem to suggest any correlation between the amount of mtDNA and the mutation. Similar results were obtained from measurements of ROS production rate and mitochondrial protein synthesis rate. All these data seemed to suggest that the presence of the mutation itself could not lead to a specific phenotype even if recent papers underline the importance of this mutation associated with declining functions in the brains of Alzheimer's patients (2).

For these reasons we started to re-sequence the entire mtDNA from all the cybrids since we thought that other mutations might co-segregate with the T414G mutation and could possibly influence the phenotype. The analysis of mtDNA is in progress but preliminary results seem to confirm the hypothesis of multiple mutations contributing to particular and complex phenotypes.

References

(1) Michikawa *et al.* (1999) *Science* **286**:774-779.
(2) Wallace *et al.* (2004) *PNAS* **101**(29):10726-10731.

367. C150T mtDNA longevity-associated polymorphism is associated with lower oxidative stress

Ai Chen, Anne Chomyn

Mitochondrial DNA (mtDNA) is highly polymorphic, and its variations in humans may contribute to individual differences in function. Recently, our laboratory has found a strikingly higher frequency of a C150T transition in mtDNA from centenarians and twins of an Italian population, and has also demonstrated that this base substitution causes a remodeling of the mtDNA 151 replication origin in human leukocytes and fibroblasts (Zhang *et al.*, 2003). To understand further the effects of the C150T transition, located in the mtDNA D-loop region, we have investigated cybrids obtained by fusing cytoplasts derived from human fibroblasts carrying the C150T transition with human mtDNA-less cells (ρ^0 cells) derived from an osteosarcoma cell line.

Because none of our fibroblasts strains are heteroplasmic, that is, none carries both 150T mtDNA and 150C mtDNA, we are unable to produce from a given individual both wild-type 150C and polymorphic 150T cybrids. Furthermore, the effects of certain mtDNA mutations and polymorphisms may be dependent on the mtDNA haplogroup. Thus, we chose for cybrid construction and analysis haplogroup-matched pairs of fibroblast strains. In particular, we used as one pair of mtDNA donors fibroblast strains of the U haplogroup. One of these, belonging to the U3a subgroup, carried the C150T transition (U3a 150T) in homoplasmic form, and the other, belonging to the U-K2 subgroup, was 100% C at position 150 (K2 wild). Another pair of fibroblasts was of the J haplogroup, one carrying the C150T transition (J2b 150T) in homoplasmic form, and the other, completely lacking the C150T transition (J1c wild). Upon analyzing three or more cybrid clones from each cytoplast fusion, we have shown clearly that the U3a 150T cybrid cell lines have higher overall respiratory capacity, and higher substrate-driven respiration rates, whether the substrate combinations were malate/glutamate, or succinate/G3P, or TMPD/ascorbate, than those of K2 wild cybrid cell lines. In addition, the U3a 150T cybrid cell lines have similar doubling times and mitochondrial membrane potential to those of K2 wild cybrid cell lines. Mitochondrial protein synthesis rates and COX II protein levels are also higher in the 150T cybrids, reflecting the same trend as the respiration capacities. In contrast, a comparison of the J2b 150T and the J1c wild cybrid cell lines showed that the J2b 150T cybrid cell lines have lower overall respiratory capacity, including the rates of malate/glutamate-driven respiration, succinate/G3P-driven respiration, and TMPD/ascorbate-driven respiration, and longer doubling times than the J1c wild-type cybrid cell lines. Mitochondrial protein synthesis rates and COX II protein levels are also lower in the 150T cybrids. Interestingly, U3a and J2b haplogroup cell lines, with the C150T transition, have a lower ROS generation rate than, respectively, K2 or J1c haplotype cell lines, which have C at position 150. In addition, we have re-sequenced the mtDNA of representative samples of each group of cybrid cell lines to rule out a contribution of mutations or novel polymorphisms to the differences in phenotype among the cybrids.

In summary, we have analyzed a number of parameters in our cell lines and have found that the 150T cell lines have in common a lower ROS production rate. This lower ROS production rate may contribute to the increased longevity associated with the presence of the C150T transition.

Reference

Zhang, J., Asin-Cayuela, J., Fish, J., Michikawa, Y., Bonafé, M., Oliveri, O., Passarino, G., De Benedictis, G., Franceschi, C. and Attardi, G. (2003) *Proc. Natl. Acad. Sci. USA* **100**:1116-1121.

Publications

Duvezin-Caubet, S., Jagasia, R., Wagener, J., Hofmann S., Trifunovic, A., Hansson, A., Chomyn, A., Bauer, M.F., Attardi, G., Larsson, N.G., Neupert, W. and Reichert, A.S. (2006) Proteolytic processing of OPA1 links mitochondrial dysfunction to alterations in mitochondrial morphology. *J. Biol. Chem.* **281**:37972-37979.

Hajek, P., Chomyn, A. and Attardi, G. (2007) Identification of a novel mitochondrial complex containing mitofusin 2 and stomatin-like protein 2. *J. Biol. Chem*. **282**:5670-5680.

Iwata, N., Zhang, N., Atzmon, G., Leanza, S., Cho, J., Chomyn, A., Burk, R.D., Barzilai, N. and Attardi, G. (2007) Aging-related occurrence in Ashkenazi Jews of leukocyte heteroplasmic mtDNA mutation adjacent to replication origin frequently remodeled in Italian centenarians. *Mitochondrion* **7**:267-272.

Walker, D., Hájek, P., Muffat, J., Knoepfle, D., Cornelison, S., Attardi, G. and Benzer, S. (2006) Hypersensitivity to oxygen and shortened lifespan in a *Drosophila* mitochondrial complex II mutant. *Proc. Natl. Acad. Sci. USA* **103**:16382-16387.

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Summary: Our laboratory has two arms: one, the basic study of immunity, the other, the application of basic knowledge to the treatment and prevention of disease.

The basic work focuses on two areas. One is our long-standing interest in NF-κB, a transcription factor that controls cellular responsiveness to many stimuli but is best known for its role in the inflammatory response to pathogens. Because it is also involved in cancer induction, regulation of NF-κB is critical and we have concentrated on regulatory issues for a number of years.

The second basic area is microRNA function. There are some 500 or more microRNAs encoded in vertebrate genomes and there is little knowledge of the precise function of many of them. We found that four are induced by inflammatory stimuli and we are now attempting to understand their precise roles and their regulation. We are also extending this interest to other microRNAs that may play roles in the immune system.

Our more applied work focuses on AIDS and cancer. These diseases are poorly controlled by the immune system and we would like to alter the system in ways that would improve its ability to counter them. Our main focus is on genetically modifying immune cells in a program we call "Engineering Immunity." This program involves using lentiviral vectors to put T cell receptor or antibody genes into hematopoietic stem cells; such genes become active when the cells differentiate into T or B cells, respectively. We are also modifying dendritic cells with genes that encode antigens. Some of these studies involve *ex vivo* infection; others involve *in vivo* infection. Most of our work has been with mice but we are now extending it to humans in a study, joint with other Los Angeles institutions, of improved T cell immunity against melanoma.

368. The outcome of HIV infection at the level of the individual cell

Alex Sigal, David Baltimore

Stochasticity (chance) is starting to be recognized as an important determinant in the outcomes of biological processes. One biological system where stochastic effects are predicted to be particularly important, based on the underlying molecular circuitry, is in HIV infection. HIV infection is cytotoxic to the infected cell. Strong evidence for this is the rapid decline in virus titer once anti-retroviral therapy is administered. This rapid decline, with a half-life of about two days, implies that when no new cells are infected, the existing pool of virus producing cells quickly disappears due to cell death. The probability of an infected cell to die *in vivo* most likely depends on the microenvironment, the cell type, and stochastic processes. A controlled environment is needed to isolate these factors. We will measure the probability of cell death in a tissue culture system, where the effects of microenvironment are minimized and the cell type is the same.

To quantify this probability, we are developing an experimental system that can detect single virus infection and cell fate at the individual cell level. This system is based on time-lapse imaging of infection at an average multiplicity of less than one virus per cell, in an incubated microscope environment.

We have assembled a time-lapse microscopy system in a fully incubated environment to quantify the timing of cell death. We have tethered T cells and macrophages to optical bottom dishes, enabling us to successfully image and track these cells. Cell death using our system is detected by morphological characteristics, including cell blebbing and apoptotic body formation. Time-lapse microscopy is performed with high resolution but low magnification objectives to obtain an acceptable signal to noise ratio without significant photo-toxicity to the cells. We have performed time-lapse experiments for over five days, and expect to prolong experiments to a week or more, a timescale significantly longer than the half-life of virus producing cells (two days).

By quantifying cell fate after the infection of a single HIV virion, we would be able to determine the probability of cell death per virion after infection. Using the tissue culture model, we would measure the variability in cell fate at a given time-point that would predominantly result from stochastic processes. This may improve our understanding of the importance of chance in cell fate after HIV infection.

Publications

Lavelle, C. and Sigal, A. (2007) Systems biology meets chromatin function: A report on the Fourth Elmau Conference on Nuclear Organization. *Chromosome Res.* **15**:247-256.

Sigal, A. *et al*. (2007) Generation of a fluorescently labeled endogenous protein library in living human cells. *Nat. Protoc.* **2**:1515-1527.

Sigal, A. *et al.* (2006) Dynamic proteomics in individual human cells uncovers widespread cell-cycle dependence of nuclear proteins. *Nature Meths.* **3**:525-531.

Sigal, A. *et al.* (2006) Variability and memory of protein levels in human cells. *Nature* **444**:643-646.

369. Regulation of inflammatory response by NF-κB dimers

Shengli Hao, David Baltimore

Inflammatory response is a double-edged sword. Although the host must clear out pathogenic insults to repair injuries, an unregulated or prolonged inflammatory response leads to a great number of diseases, including AIDS, autoimmune diseases such as type I diabetes, multiple sclerosis (MS), cancer, and vascular diseases. Therefore, the effective prevention and treatment of these diseases relies on a deep understanding of how inflammatory response is controlled in the body. NF-κB is a key regulator for inflammatory response by directly controlling expression of a great number of molecules critical for this process. It functions as a homodimer or heterodimer composed from five members: RelA, RelB, c-Rel, p50 and p52. Moreover, exploration of the specific property and function of each dimer is crucial to understanding and ultimately discovering the effective treatment of this disease.

The goal of this study is to find how each dimer regulates inflammatory response and to obtain an accurate picture of how inflammatory response is controlled at different stages. We are focusing primarily on the p50 homodimer. Since p50 can form homodimers as well as heterodimers with other four NF-κB members in the cells, it is impossible to analyze the function of the p50 homodimer using a traditional genetic approach. To overcome this, last year we generated a p50 mutant that forms only homodimers; it also serves as a fully functional transcription factor.

Although the p50:p65 heterodimer is the protype NF-κB dimmer and has been extensively studied, there is a significant amount of p50 homodimer in the nucleus, especially in various diseases, e.g., some tumor cells. It has been considered a gene repressor because it lacks consensus transactivation domain. However, this has not been tested via experimentation. Our p50 homodimer mutant (designated as p50*) makes it possible to address this question. Using the lentiviral vector system, we successfully introduced p50* into the wild-type mouse embryonic fibroblast cell line with different expression levels. Then, we proved p50* can suppress gene activation upon stimulation by TNF.

Using Affymatrix Microarray 2.0 for the whole mouse genome analysis, we discovered the p50 homodimer serves as an activator that enhances both the basal level and TNF induction of a gene subset. This result was confirmed by qPCR. Using a short hairpin RNA to knock down the IkB family member Bcl3, we found this activating effect of the p50 homodimer occurs partly through interaction with Bcl-3.

370. Analysis of regulation and function of microRNAs of miR-146 family

Konstantin D. Taganov, Mark P. Boldin, David Baltimore

Inflammation is a complex, highly regulated defense reaction orchestrated by the host in response to an invading pathogen or injury. The conserved pathogen molecules are sensed by the innate immune cells such as macrophages and dendritic cells via a limited number of pattern-recognition receptors of the Toll-like receptor (TLR) family. Once activated through their TLRs, innate immune cells orchestrate the immune response aimed at the containment and destruction of the pathogen as well as the activation of the adaptive immune system. TLR signaling has layers of negative regulation, since its overactivation can be deleterious to the organism and is the cause of multiple systemic and local inflammatory and autoimmune diseases.

MicroRNAs (miRNA) represent a class of tiny (~22 nt) endogenous RNAs found in most metazoan organisms as well as in viruses. Owing to their ability to regulate expression of almost any protein-coding target gene, miRNAs are implicated in a variety of biological processes in animals. Recently, we have carried out a systematic effort to identify miRNAs that might play a role in the mammalian response to bacterial infection [1]. We identified three miRNAs that are sharply upregulated (miR-146, miR-132 and miR-155) in response to LPS treatment in monocytic cells, and performed a detailed analysis of miR-146 expression upon challenge with various microbial components and proinflammatory cytokines. Our findings suggest that miR-146 may function as a novel negative regulator of immune receptor signaling through post-transcriptional regulation of two key adapter molecules downstream of Toll-like and IL-1 receptors, TRAF6 and IRAK1.

Human genome, as well as most mammalian genomes, contains two mir-146 genes located on chromosome 5 (miR-146a) and 10 (miR-146b). We observed significant up-regulation of miR-146b primary transcript in response to LPS treatment, while its corresponding pre-miR and mature forms, in contrast to miR-146a, were below the detection level, suggesting that processing of this microRNA is controlled by different mechanism. Thus, mature miR-146a/b have distinct modes of regulation at levels of processing and transcription, and considering the nucleotide sequence difference between them, might contribute to the gene regulation in a very complex way. To advance our knowledge of the physiological role of miR-146 family in immune signaling, we plan to examine the contribution of these miRNAs to the development of various hematopoetic cell lineages as well as to the immune response against bacterial pathogens in miR-146a and mir-146b knockout mice that we are generating. We plan to complement this work by gain-of-function studies in transgenic mice overexpressing miR-146a in various hematopoietic compartments as well as by bone marrow transfer experiments.

Reference

Taganov, K., Boldin, M., Chang, K. and Baltimore, D. (2006) *PNAS* **103**(33):12481-12486.

371. Methylation of NF-κB by Smyd2

Chee-Kwee Ea, David Baltimore

NF-κB undergoes several post-translational modifications upon activation, which include phosphorylation, ubiquitination, nitrosylation and acetylation. These regulatory modifications have distinct functional consequences. For example, acetylation of RelA at K218 and K221 inhibits IkBα binding and enhance DNA-binding, while acetylation of RelA at K122 and K123 inhibits its transcriptional activity. Protein methylation is another interesting form of post-translational modification. Histones are one of the best-studied proteins that undergo methylation. Specific sites of methylation on histones correlate with either activation or repression of transcription. Recently, several transcription factors, including p53, STAT1, and RARα, have been shown to be methylated. Thus we speculate that NF-κB may be regulated by methylation.

The major goal of this project is to determine if RelA is methylated in response to stimulation. We first test if RelA can be methylated *in vitro*. We have established an *in vitro* methylation assay using recombinant RelA protein as a substrate. To this end, we found that RelA is methylated by Smyd2 *in vitro* at the N-terminus. Smyd2 belongs to a subfamily of SET domain containing methyltransferases with a unique domain architecture. This family of proteins is defined by a SET domain that is split into two segments by an MYND domain, followed by a cysteine-rich post SET domain. We further shown that overexpression of Smyd2 enhance the expression of TNF1-induced NF-κB reporter, and the methyltransferase activity of Smyd2 is required. These results set the stage for a detailed study of the biological function of RelA methylation.

372. Molecular mechanisms of modulation of TNF receptor signaling by A20 and B94 proteins

Mark P. Boldin, Konstantin D. Taganov, David Baltimore

Members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily play a crucial role in activation, proliferation, survival and death of cells in the immune system. The prototype death receptor of the family is the type I TNF receptor (TNFR1). The biological functions triggered by the TNFR1 are characterized by a remarkable duality – infliction of tissue damage and cell death goes hand in hand with activation of tissue repair and expansion. The physiological reason for this duality lies in the ability of this receptor to trigger two kinds of intracellular signaling programs: a proteolytic cell death cascade and a number of kinase cascades leading to activation of gene expression, some of which may protect cells against cytotoxicity. TNF activates hundreds of genes at the transcriptional level of which a significant portion encodes intracellular modulators of the TNF receptor signaling or regulators of crosstalk between the TNF receptors and other signaling systems. Molecular mechanisms of action of two such regulatory molecules, called A20 and B94, are at the focus of our research.

While much knowledge has been accumulated in the past about activation of the core TNF signaling pathways, very little is known about their regulation and termination. A20 is a TNF-inducible gene that works in a negative feedback loop fashion and inhibits the TNFR1 signaling. We are currently trying to understand the molecular mechanism of A20 action and its target(s) in the TNF signaling pathways using a combination of proteomics and molecular biology approaches. We have recently discovered that the C-terminal part of A20 molecule, composed of seven zinc-fingers, represents a novel type of ubiquitin-binding domain that can interact specifically with Lysine-63 (K63)-linked polyubiquitin chains. Polyubiquitin chains assembled through Lysine-48 (K48) of ubiquitin act as a signal for substrate proteolysis by 26S proteasomes, whereas chains assembled through K63 play a nonproteolytic role in activating NF-kB signaling, DNA repair and protein trafficking. We found that A20 molecule binds and sequesters K63-linked polyubiquitin chains on a key adapter molecule in TNF receptor signaling, RIP1, and thus prevents the sequential activation of downstream IKK kinase complex.

B94 is an intracellular protein, which, like A20, is induced by TNF and other proinflammatory stimuli. Our preliminary findings indicate that B94 can act as a positive modulator in the NF-κB activation pathway. We plan to use B94 RNAi knockdown cell lines and B94 gene knockout mice to clearly establish the physiological role of this gene. We are also addressing the question of the molecular mechanism of action of B94 utilizing a combination of yeast two-hybrid and proteomics techniques.

373. Engineering immunity to treat cancer

Lili Yang, Joy Tseng, Abigail Elliott, David Baltimore

The concept of Engineering Immunity is to harness the immune system to treat diseases. We have developed a method to genetically program hematopoietic stem cells (HSCs), the common immune cell progenitors, to develop into T cells with the desired specificity *in vivo*. In a mouse tumor model, we were able to show that this method could generate large number of anti-tumor T cells that resulted in efficient tumor resistance and eradication (Reference #1). To further advance and mature this method, we have been actively working on the following areas:

(1) Engineering immunity to treat melanoma

In July 2005, we started a collaboration with several research and clinical groups at UCLA, USC, CHLA and UCHC to move the Engineering Immunity method into clinical trials to treat melanoma. A Phase I clinical trial is planned to launch at the end of 2007

at UCLA, conducted by Drs. Anthony Ribas and James Economou's clinical team.

(2) Generation of antigen-specific T cells with enhanced function *in vivo*

As an extension of our Engineering Immunity concept, we have been working on the generation of antigen-specific T cells with enhanced function *in vivo*. Taking advantage of our lab's extensive experience on engineering viral vectors, we are able to construct vectors that efficiently co-express multiple genes under the control of a single promoter. This technique allows us to put into one vector the two genes encoding an antigen-specific TCR, together with a regulatory gene that can endow a desired function to a T cell. One of such genes we tested is IL-15, which has been considered to play an important role on CD8 cytotoxic T cell homeostasis and memory. In a mouse tumor model, we proved that this strategy generated memory-phenotype anti-tumor T cells of an expanded colony size, and effectively protected mice from tumor challenge.

(3) Targeted gene delivery to genetically modify HSCs *in vivo*

As a hallmark of gene therapy, targeted gene delivery *in vivo* can greatly facilitate the application of gene therapy, including our "Engineering Immunity" strategy. To this end, we have developed a gene delivery system to transfer genes to chosen cell types (Reference 2). In particular, we have generated lentiviruses that can specifically infect cells expressing mouse c-kit, a marker for mouse HSCs; and human CD34, the sole reliable marker for human HSCs. We have proved this system to be highly specific and efficient, by targeted infection of cell lines expressing the c-kit or CD34 marker. The targeted infection of primary mouse HSCs and human HSCs are under testing, both *in vitro* and *in vivo*. Other applications of this system include the *in vivo* correction of blood cell genetic deficiencies, holding potential for treating diseases such as SCID and the X-linked autoimmunity.

(4) *In situ* dendritic cell vaccination

Dendritic cell (DC) vaccination has been broadly used to mount T cell immunity against cancer and infectious diseases. Genetically modified DCs that express tumor or viral antigens are considered to be powerful vaccines. However, the current protocol involves the costly and tedious process including collect peripheral blood from a patient, culture DC *in vitro*, transduce the cells with vectors, and infuse the cells back to the same patient. Falling into the category of personal medicine, the process is costly, tedious and un-consistent. We developed a novel method of *in situ* DC vaccination. Our strategy is to engineer the tropism of a lentiviral vector to make it infect only DCs, but not any other tissue cells. Using Chicken Ovalbumin (OVA) as a model antigen, we were able to induce a strong anti-OVA T cell response and memory in a mouse via a single shot of such lentivector encoding OVA. What's more, by modifying the expression pattern of OVA, we can also induce a strong antibody response in mouse. In the tumor challenge experiments, all mice received such vaccination showed complete resistance to OVA tumor challenge. Currently we are in testing of the method for authentic tumor antigen, in a B16 mouse melanoma model. This new method could potentially be used to replace the current DC vaccination approaches, by a single shot of a common lentivector stock, to treat a broad range of diseases including cancer and HIV.

373a. Engineering immunity to treat HIV and other dangerous pathogens

Xin Luo, Ryan Michael O'Connell, Eun Mi Hur, Kenneth Yu, Anthony Walls, Emily Maarschalk, Christine Kivork, Angie Frausto, Lili Yang, David Baltimore

Supported by Bill and Melinda Gates Foundation through the Grand Challenges in Global Health Initiative, we are exploring a fundamentally new way of stimulating the immune system to fight off infectious diseases, focusing on HIV as a test of the concept. The promise of this project is that for some infections, including HIV, the immune system's natural responses are inherently inadequate, and therefore the traditional approach of using vaccines to stimulate and boost these responses is likely to be ineffective. As an alternative, we propose to genetically engineer immune cells that can produce adequate responses. Our work is intended to eventually lead to immunotherapy for people who are infected with HIV. It could also lead to new ways to prevent HIV infection.

Our strategy is to use gene therapy for expression of genes encoding neutralizing antibodies against HIV. Multiple problems will be solved to allow expression of purposely-designed antibody genes in the cells of infected people. We plan to implant genes in blood stem cells and allow the cells to give rise to B cells, the body's natural antibody-producing cells, requiring the solution of particular problems posed by the architecture of antibody genes. We must also design antibodies or antibody-like proteins that can efficiently neutralize the infectivity of HIV. This will be done by design methods or by selection. We must then prove the effectiveness of the design in human cells or in a mouse/human chimera. Finally, we have to drive the cost of the process to a low enough level to use in the less developed world. At low cost, it could even be a vaccine.

With the goal of developing this general method, captured by the phrase “Engineering Immunity” to treat infection by HIV and other dangerous pathogens, we have the following projects:

Project 1

To perfect lentiviral expression systems that can impart the anti-HIV specificities to the mouse antibody repertoire

Lili Yang, Anthony Walls

The Baltimore laboratory has devised methods to generate functional T cells of defined antigen specificity in mice by retrovirus-mediated expression of T cell receptor genes in hematopoietic precursor cells (Reference #1). We will adapt these methods to generate functional B cells capable of secreting antibodies (Abs) or Ab-like proteins upon stimulation with antigen. Using a mouse model antibody/antigen system, we have constructed lentiviral vectors that co-express a membrane-bound form of a lysozyme-specific Ab, and a secreting form of anti-HIV neutralizing Abs (Nab). Upon transferring into HSCs, such a lentivector will direct the generation of "dual-specific" B cells in mouse that can go clonal expansion upon injection of lysozyme, and secreting high titers of anti-HIV Abs. So far we have achieved the generation of lysozyme-specific B cells in mouse. Functional analysis of these B cells in response to antigen stimulation *in vitro* and *in vivo* is ongoing.

Project 2

To perfect lentiviral expression systems that can impart the anti-HIV specificities to the human antibody repertoire

Kenneth Yu

A natural B cell can produce both membrane-bound and secreting antibodies of a single specificity, and of a particular isotype, through molecular mechanisms called alternative splicing and isotype switching. The elegant genetic machinery controlling these mechanisms is located within the 1Mb-length immunoglobulin heavy chain loci. Our goal is to incorporate the minimal necessary genetic elements of this machinery into a lentivector (the maximal capacity is 10kb) to achieve the control of an antibody gene expression in a natural B cell manner. Using the engineering immunity approach, we expect to transduce human CD34+ HSCs with such a lentivector and program the HSCs to develop into fully normal HIV-specific B cells that can respond to HIV antigen, undergo alternative splicing and isotype switching, and secrete high titer of anti-HIV Nabs. The engineered lentivectors will be tested *in vitro* in a human B lymophopoiesis culture system (described in Project 3), and *in vivo* in a human immune system (HIS) mouse model (described in Project 4). Currently, we are testing the vector system to undergo alternative splicing in inducible B cell lines and developing the virus production methodology that will enable high titer production of lentiviral vectors that contain RNA processing signals.

Project 3

To construct anti-HIV-producing lentiviral vectors and test *in vitro* using a human B lymphopoiesis culture system

Xin Luo, Emily Maarschalk

Based on the reported long-term, *in vitro* culture system initiated with $CD34^+$ human cord blood hematopoietic progenitor cells that supports normal human B-lineage development (Fluckiger *et al*., 1998), we have started a two-stage system in order to perfect the conditions required for B lymphopoiesis *in vitro* and subsequently, for future testing of lentiviral constructs that drive the transduced human HSCs to differentiate into human B cells with dual specificity *in vitro*. The first stage is to generate an adequate supply of $CD19^+$ B cells from $CD34^+$ human hematopoietic progenitor cells. Using a MS-5 stroma cell co-culture system in the presence of growth factors, we have achieved the generation of CD19+ cells. We further proved that we can transduce the CD34+ cells with a reporter lentivector at high efficiency (>80%), and differentiate the transduced cells into CD19+ cells. The second stage is to activate naïve $CD19^+$ B cells and differentiate them into Ab-producing plasma cells. By stimulating naïve B cells with mitogens in the presence of T cell help, we observed the class switching and upregulation of B cell activation markers. By transducing the HSCs with lentiviral constructs carrying HIV-specific neutralizing antibody genes, we have succeeded in programming mature B cells and plasma cells to express one of the antibodies, b12, *in vitro*. The level of b12 production from plasma cells is not yet optimal; thus, we have cloned an immunoglobulin light chain promoter whose activity is specifically heightened in a plasmacytoma cell line. Whether this promoter can increase b12 production in normal plasma cells will be examined.

Project 4

To construct anti-HIV-producing lentiviral vectors and test *in vivo* using a HIS (Human Immune System) HIV infection mouse model

Ryan Michael O'Connell, Christine Kivork, Angie Frausto

The Human Immunodeficiency Virus (HIV) is a retrovirus and the causative agent of HIV/AIDS. Since its emergence over two decades ago, approximately 20 million people have died from HIV, while another 40 million are presently living with an infection. Despite the enormous amount of research that has taken place since the discovery of this virus, an effective vaccine against HIV has not yet been realized.

One major obstacle preventing the advancement of vaccines against HIV has been the lack of small animal models of infection to test novel approaches. Manz and co-workers have recently demonstrated that intrahepatic injection of human $CD34^+$ cord blood stem cells in newborn $RAG2^{-/-}g_c^{-/-}$ mice results in reconstitution of a human adaptive immune system in mice (Traggiai *et al.*, 2004). The transplanted, or Human Immune System (HIS), mice develop human B, T and dendritic cells, generate structured primary and secondary lymphoid organs, and establish a functional immune response. Importantly, HIS mice are susceptible to infection by HIV.

We have adopted the HIS model in our laboratory, and also observe strong engraftment of CD34+ cord blood stem cells in the bone marrow, and development of human B and T lymphocytes in the periphery. Furthermore, we have successfully delivered

reporter genes to CD34+ cord blood stem cells using lentiviral vectors, and subsequently observed strong and long-lasting reporter gene expression in mature B lymphocytes in HIS mice reconstituted with transduced stem cells. This model will provide us with a convenient and straightforward *in vivo* system to evaluate the efficacy of specific anti-HIV antibodies produced by human B cells to neutralize HIV infection. We are currently working to achieve high levels of antibody expression in our HIS mice, and to establish protocols that will result in consistent and detectable HIV infections that can be analyzed in response to different antibody treatments.

Project 5

To construct lentiviral vectors encoding IgA isotype of anti-HIV neutralizing antibodies and study their application to treat HIV infection

Eun Mi Hur

Since mucosal transmission of virus is the major route of HIV infection in human, establishing mucosal protection with effective neutralizing antibodies secreted to mucosal sites such as IgA is one of the objectives in this study. To accomplish this objective, we planned to engineer broadly neutralizing anti-HIV antibodies 4E10, 2F5, 2G12, and b12 in human IgA form which is secreted to mucosal sites including genital, gastrointestinal and bronchial tracts. The animal model used in this study is an immuno-compromised mouse reconstituted with human immune system (HIS mouse model). This mouse model allows to produce anti-HIV antibodies in human IgA form *in vivo* and to secrete them to mucosal sites that are the primary location of HIV transmission. In this model system, it is prerequisite to test whether human IgA is efficiently transported through mouse polymeric immunoglobulin receptor (poly-Ig-R) to mouse epithelium. We have tested the efficiency of inter-species transport of human IgA into mouse mucosal sites and confirmed the efficiency of transport is in comparable range to mouse IgA transport. Three broadly neutralizing anti-HIV antibodies, b12, 2G12 and 2F5 in human IgA2 form have been cloned in eukaryotic expression vector. The expression of these antibodies has been confirmed in human cell line. Gene transfer and expression of these antibodies in human lymphocytes and HSC using lentiviral vector are ongoing.

Project 6

To engineer bispecific and other designer anti-HIV antibodies and evaluate them for binding to and neutralizing HIV

Anthony West Jr.[*], *Joshua Klein*[*], *Priyanthi Peiris*[*], *Pamela Bjorkman*[**], *David Baltimore*

Using protein engineering and design methods, we are attempting to make protein reagents that will be more potent than existing neutralizing antibodies. This will allow them to be effective at lower concentrations in the blood or in secretions. We are pursuing several approaches, such as making bispecific Abs containing two different combining sites. We are expressing, purifying and determining affinities and an *in vitro* neutralization potencies of the following Abs and Ab-like proteins: bispecific antibodies with specificities for two different HIV epitopes, chimeric proteins containing the gp120-binding domains of CD4 linked to a CD4-induced antibody specificity, and multivalent antibodies. For further information please see Bjorkman lab abstracts.

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Project 7

To engineer the lentivector delivery system for the application of Engineering Immunity approach

Leslie Bailey[*], *Lili Yang, Pin Wang*[*], *David Baltimore*

We have identified an efficient and flexible method to target lentivirus-mediated gene transduction to a pre-determined cell type (Reference #2). It involves incorporation of a cell-specific binding determinant (CBD) and a fusogenic protein as two distinct entities into the lentiviral surface. The fusogen is modified so that it will not bind to its cognate receptor, so the CBD determines the targeting specificity. Using CD20 as a target antigen for human B cells, we have demonstrated that this targeting strategy is effective both *in vitro* and in intact animals. This methodology is flexible and can be extended to other forms of cell type-specific recognition to mediate targeting. The only requirement is that the antibody (or other binding protein) must be endocytosed after interaction with its cell-surface binding determinant. The technique allows for targeting of gene therapy by direct injection of a vector.

Hematopoietic stem cells (HSCs) are capable of differentiating into the entire blood system including cells important for the immune system and can provide a life-long source of a therapeutic gene. We have used our targeting method to target CD34, the sole reliable marker for HSCs. By displaying an anti-CD34 antibody as well as the fusogenic protein on the viral surface we were able to efficiently and selectively transduce CD34+ cells lines in *vitro* and *in vivo* in a xenografted tumor mouse model. We are further testing the ability of these engineered lentivectors to transduce primary CD34+ cells *in vitro* and *in vivo* in the Rag2-/-γc-/- HIS mouse model. Our future plan is to use the CD34-targeting lentivectors to delivery anti-HIV neutralizing antibody genes into HSCs to treat HIV infection.

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(This project is a joint effort with the following groups: The laboratories of David Baltimore and Pamela Bjorkman at Caltech, and Pin Wang's group at USC.)

References

Yang L. and Baltimore D. (2005) *Proc. Natl. Acad. Sci. USA* **102**(12):4518-4523.

Yang L., Bailey L., Baltimore D. and Wang P. (2006) *Proc. Natl. Acad. Sci. USA* **103**(31):11479-11484.

Publications

An, D.S, Donahue, R.E., Karnata, M., Poon, B., Metzger, M., Mao, S.H., Bonifacino, A., Krouse, A.E., Darlix, J.L., Baltimore, D., Qin, F.X.F. and Chen, I.S.Y. (2007) Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc. Natl. Acad. Sci. USA* **104**(32):13110-13115.

An, D.S., Qin, F.X.F., Auyeung, V.C., Mao, S.H., Kung, S.K.P., Baltimore, D. and Chen, I.S.Y. (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol. Therapy* **14**(4):494-504.

Baltimore, D. (2006) Science for life: A conversation with Nobel laureate David Baltimore. Interview by Barbara J. Culliton. *Health Aff. (Millwood)* **25**(4):W235-240.

Hoffmann, A. and Baltimore, D. (2006) Circuitry of nuclear factor kappaB signaling. *Immunol. Rev.* **210**:171-186.

O'Connell, R.M., Taganov, K.D., Boldin, M.P., Cheng, G.H. and Baltimore, D. (2007) MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. USA* **104**(5):1604-1609.

Sen, R. and Baltimore, D. (2006) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *J. Immunol.* **177**(11):7485-7496; *Cell* (1986) **46**:705-716.

Taganov, K.D., Boldin, M.P. and Baltimore, D. (2007) MicroRNAs and immunity: Tiny players in a big field. *Immunity* **26**(2):133-137.

Taganov, K.D., Boldin, M.P. *et al.* (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. USA* **103**(33):12481-12486.

Yang, L.L., Bailey, L., Baltimore, D. and Wang, P (2006) Targeting lentiviral vectors to specific cell types *in vivo*. *Proc. Natl. Acad. Sci. USA* **103**(31):11479-11484.

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Summary: My laboratory is interested in protein-protein interactions, particularly those mediating immune recognition. We use X-ray crystallography and biochemistry to study purified proteins, and confocal and electron microscopy to examine protein complexes in cells. Some of our work focuses upon homologs and mimics of class I major histocompatibility complex (MHC) proteins. Classical class I MHC proteins present peptides derived from self and non-self proteins to T cells during immune surveillance. MHC homologs share similar three-dimensional structures with classical MHC molecules but have different functions, including immune functions (IgG transport by FcRn, the neonatal Fc receptor, and evasion of the immune response by viral MHC mimics) and non-immune functions (regulation of iron or lipid metabolism by HFE and ZAG, and serving as a chaperone for pheromone receptors in the case of the M10 proteins). We are also comparing the structures and functions of host and viral Fc receptors with FcRn.

Transfer of maternal IgG (immunoglobulin G) molecules to the fetus or infant is a mechanism by which a mammalian neonate acquires humoral immunity to antigens encountered by the mother. The protein responsible for the transfer of IgG is the MHC class I-related receptor FcRn. MHC class I molecules have no reported function as immunoglobulin receptors; instead they bind and present short peptides to T cells as part of immune surveillance to detect intracellular pathogens. We solved the crystal structures of rat FcRn both alone and complexed with Fc. We are now using information obtained from our crystallographic and biochemical studies to determine how FcRn-IgG complexes are transported across polarized epithelial cells. We are using a combination of confocal and electron microscopy to study the itineraries of FcRn-containing endosomes in transfected epithelial cells and in the proximal small intestine of neonatal rats. Using electron tomography and a new ligand labeling/identification protocol, we derived a 3D map of transport vesicles in neonatal intestinal epithelial cells at 4-6 nm resolution. We are also doing structure/function studies of other Fc receptors that are not MHC homologs: gE-gI, a viral Fc receptor for IgG; FcaRI, a host receptor for IgA; and the polymeric immunoglobulin receptor (pIgR), which transports dimeric IgA and polymeric IgM into secretions.

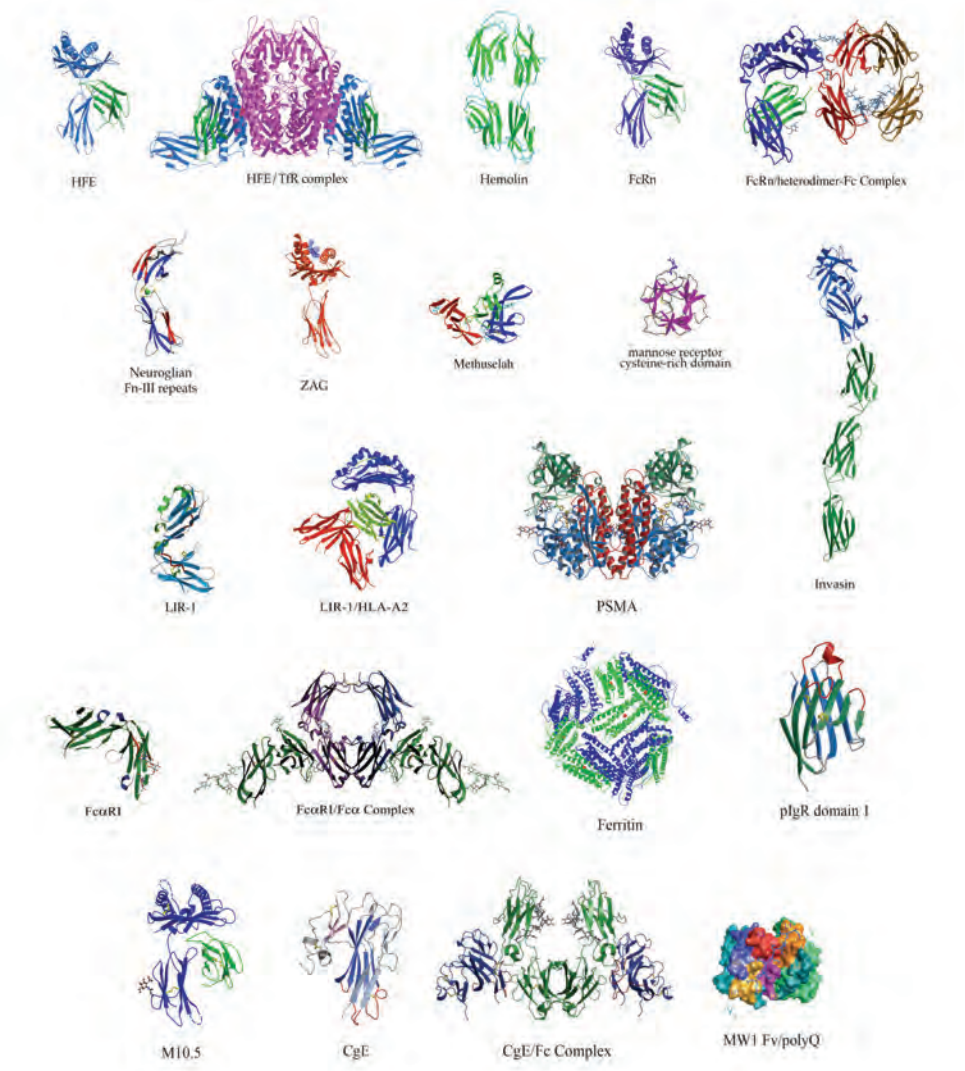
HFE, a recently discovered class I MHC homolog, is involved in the regulation of iron metabolism, an unexpected function for an MHC-related protein. HFE was discovered when its gene was found to be mutated in patients with the iron-overload disease hereditary hemochromatosis. With the demonstration that it binds to transferrin receptor (TfR), the receptor by which cells acquire iron-loaded transferrin, HFE has been linked to iron metabolism. We have solved crystal structures of HFE alone and HFE bound to TfR. The interaction of HFE with TfR is a fascinating system to study because we can use crystal structures to determine how binding of HFE interferes with transferrin binding, if conformational changes in the receptor are involved in the binding of either transferrin or HFE, which part of the MHC-like HFE structure binds TfR, and how the HFE interaction with TfR compares with interactions of ligands with MHC and MHC-like (e.g., FcRn) proteins. We are using confocal microscopy and other imaging techniques to investigate HFE and TfR intracellular trafficking in transfected cell lines. In addition, we are exploring the roles of other molecules, such as ferroportin and hemojuvelin, in the regulation of iron metabolism.

We are also interested in other MHC homologs, including proteins encoded by viruses. Both human and murine cytomegalovirus (HCMV, MCMV) express a relative of MHC class I heavy chains, probably as part of the viral defense mechanism against the mammalian immune system. Our biochemical studies show that the HCMV homolog associates with endogenous peptides resembling those that bind to class I MHC molecules. We recently solved the crystal structure of the HCMV homolog bound to a host receptor protein, which we are comparing to our structure of the same receptor bound to a human MHC protein. Surprisingly, the viral MHC homolog contains a peptide-binding groove that includes virtually all the features of grooves in classical host class I MHC molecules and is occupied with a peptide. Our HCMV homolog/receptor co-crystal structure does not answer the question of why the viral MHC homolog binds peptides, however, because the receptor-binding site is distant from homolog's peptide-binding groove. We hope to use this structural information to understand how peptide binding by a class I MHC mimic is used in viral evasion of the host immune response.

Recent studies from other laboratories have revealed expression of a family of class Ib MHC proteins

(M10s) that interact with putative pheromone receptors in the rodent vomeronasal organ. This interaction may play a direct role in the detection of pheromonal cues that initiate reproductive and territorial behaviors. Our crystal structure of M10.5 shows that M10 proteins fold into a structure similar to that of a *bona fide* class I MHC molecule. Unexpectedly, however, the M10.5 counterpart of the MHC peptide-binding groove is open and unoccupied, revealing the first structure of an empty class I MHC molecule. Our biochemical data suggest that M10.5 associates with some sort of groove occupant, most likely non-peptidic. The challenge now is to discover the physiological ligands of M10 proteins and understand how they associate with pheromone receptors to influence mating behaviors.

Our structural work on class I MHC homologs has elucidated new and unexpected recognition properties of the MHC fold. For FcRn and HFE, the structural and biochemical studies have revealed a similar fold and common properties, including the assumption that both receptors "lie down" parallel to the membrane when binding ligand, and a sharp pH-dependent affinity transition near neutral pH. In the case of FcRn, we have elucidated the structural basis of its pH-dependent interaction with IgG and are now focusing upon cell biological studies of intracellular trafficking, for which the pH-dependent interaction is critical. The pH dependence of the HFE-TfR interaction suggested to us that intracellular trafficking studies of HFE would be interesting so much of our future efforts on both the FcRn and HFE systems will involve probing their function in a cellular context using imaging techniques. Our functional studies of M10 proteins are just beginning, since we do not understand the nature of the interaction between an M10 protein and a pheromone receptor and how that relates to binding to an as yet unidentified groove occupant. Our functional studies of viral MHC homologs are somewhat further along, in that we have identified the groove occupant to be peptide(s), but do not yet understand the significance of peptide binding in the function of the viral MHC homolog or the life cycle of the virus.



374. Characterization of FcRn-mediated transport pathways via confocal microscopy

Devin B. Tesar, Galina Jerdeva

Movement of specific protein molecules across epithelial cell barriers by their cognate receptors is achieved via a multivesicular transport pathway known as transcytosis. Discrete steps in the procession of a receptor-bound ligand through the transcytotic network are characterized by association with distinct subpopulations of endosomal compartments. These subpopulations of endosomes can be identified by confocal microscopy using fluorescent markers (such as fluorescently-labeled transferrin) or antibodies against such markers (such as anti-Rab antibodies). We are currently working to decipher the transcytotic itinerary of FcRn, with or without its IgG ligand, by colocalizing FcRn and IgG with different endosomal markers. To achieve this, FcRn can be visualized by staining with an anti-FcRn monoclonal antibody that is directly conjugated to Alexa488. Rat IgG or Fc fragment can be directly labeled with fluorescent dyes (such as the Alexa 488 or 568), and antibodies against specific endosomal markers can be viewed using secondary antibodies conjugated to a far-red dye (such as Alexa 647). This allows for three-color confocal analysis to determine the intracellular localization of FcRn, its ligand, and marker proteins for particular compartments at different stages within the transport process. More recently, we have been working to develop techniques for live cell imaging using fast confocal imaging and have acquired an UltraVIEW ERS spinning disk confocal system. Spinning disk technology is specifically suited for fast acquisition of events in living cells that happen beyond the resolution of conventional confocal microscopes. The use of a highly sensitive CCD detector allows for fast acquisition under conditions of limited laser exposure, greatly increasing fluorophore lifetime over the course of an experiment. The use of a Piezo-controlled objective turret allows for fast and precise Z-stepping in increments of 50 nm, making fast acquisition of Z-stacks possible. Standard microscopes, despite having exceptional resolution for imaging of fixed specimens, require approximately 7-15 seconds to scan a single channel at a resolution comparable to what a spinning disc system can accomplish in 20-50 milliseconds of exposure. These data can then be compared to the transport pathways of more well characterized receptors such as the polymeric Ig receptor (pIgR) to evaluate which steps might be unique to, or particularly important during FcRn-mediated transport of IgG.

375. Identification of effector molecules that mediate the trafficking of FcRn in cells

Devin B. Tesar, Johannes Graumann[*]

The intracellular trafficking of a receptor and its ligands requires the coordinated association of the receptor cytoplasmic tail with effector molecules responsible for regulating and executing specific steps of the trafficking pathway. Determinants within the cytoplasmic tail of a given receptor confer the specificity of these interactions. While many of the molecular components of the cytosolic trafficking machinery are very well conserved across different organisms, different receptors may utilize different components while being directed to their target destinations. We wish to determine the identity of effector proteins associated with FcRn during its intracellular trafficking itinerary. FcRn obtained from both natural sources (neonatal rat intestine) and tissue culture cells (transfected MDCK cells) is co-immunoprecipitated using FcRn-specific antibodies, and the co-precipitating proteins are subjected to Multidimensional Protein Identification Technology (MudPIT) analysis. MudPIT allows mass spectrometric analysis of a complex protein mixture by coupling a two-dimensional chromatographic separation of the mixture to direct downstream injection of the sample into the mass spectrometer. Identified targets will be verified by performing pull-downs of the identified binding partner and demonstrating the co-precipitation of FcRn. Further experiments will then be carried out to assess the role of these targets in FcRn-mediated intracellular trafficking.

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376. Trafficking studies of the chicken yolk sac IgY receptor

Devin Tesar, Evelyn Cheung

In mammals, the transfer of passive immunity from mother to young is mediated by the MHC-related receptor FcRn. FcRn binds to the Fc region of IgG tightly at the acidic pH of endosomes and releases the IgG at the slightly basic pH of blood, allowing transport of IgG across epithelial cell barriers. In avian species, maternal IgY present in egg yolk is transferred across the yolk sac *in ovo* to provide the chick with passive immunity during gestation and early independent life. In a previous study, the chicken yolk sac IgY receptor (FcRY) was isolated from yolk sac membranes and shown to be unrelated to FcRn or class I MHC molecules, but rather a homolog of the phospholipase A_2 receptor, a member of the mannose receptor family. Here we provide the first demonstration that FcRY functions in endocytosis, transcytosis and recycling of chicken FcY/IgY when expressed in rat inner medullary collecting duct (IMCD) cells, a polarized epithelial cell line. Confocal immunofluorescence studies showed that FcRY extensively colocalized with endocytosed IgY in subcellular compartments when incubated with IgY at pH 6, but not pH 8, mimicking the pH-dependent uptake of IgG by FcRn in other studies. We also show that FcRY-mediated internalization occurs via clathrin-coated pits, and that internalized IgY is trafficked to EEA1-positive early endosomes and Rab11-positive recycling endosomes. Quantitative internalization and transport assays demonstrated that FcRY can bi-directionally transcytose and recycle labeled ligands from either the apical or basolateral surface. Internalization and transcytosis occurred at pH 6, but not at pH 8, and could be saturated by the addition of a 200-fold excess of unlabeled IgY. The microtubule depolymerizing agent nocodazole partially inhibited transcytosis in both directions, but did not have a significant effect on recycling, indicating that these processes rely on different components of the intracellular trafficking machinery. Taken together, these data represent the first cell biological

evidence that FcRY and FcRn are structurally distinct, but functionally equivalent molecules, and provide an intriguing example of how evolutionary forces can give rise to systems in which similar biological requirements in different species are satisfied utilizing distinct protein folds.

377. Characterization and visualization of the FcRn-dependent transcytotic pathway using high-resolution fluorescence confocal microscopy

Galina V. Jerdeva, Devin B. Tesar, Scott E. Fraser[*]

Specific delivery of proteins across polarized epithelia is controlled by receptor-mediated transcytosis. Based on studies of the trafficking of model receptors such as the polymeric immunoglobulin receptor (pIgR), the pathways for receptor-mediated transport of protein ligands in the basolateral to apical direction are relatively well understood. Less is known about the trafficking pathways for receptors that transport from the apical to basolateral surface. One such receptor is the neonatal Fc receptor (FcRn), which transports maternal immunoglobulin G (IgG) across intestinal or placental epithelial barriers to provide immunity to fetal or newborn mammals and serves as a protection receptor for IgG, thereby increases its serum half-life. To investigate FcRn-mediated transport of IgG, we are using Madin-Darby Canine Kidney (MDCK) cells stably expressing FcRn (MDCK-FcRn). The transfected cells specifically transcytose IgG and Fc across polarized cell monolayers, presenting a system that mimics the *in vivo* FcRn-dependent transport system. Using high-resolution confocal and multiphoton microscopy, we will identify intracellular compartments involved in transcytosis of labeled Fc and IgG in MDCK-FcRn cells by colocalization studies with organelle-specific markers. Comparing the intracellular trafficking of FcRn and its ligands with trafficking of a basal to apical model receptor, pIgR, which transports dimeric dIgA (dIgA), will provide information about common and specific trafficking pathways of IgG versus dIgA. To compare FcRn- and pIgR-dependent intracellular pathways**,** we have generated MDCK cells expressing both receptors (MDCK-FcRn-pIgR) to track simultaneous internalization of fluorescently-labeled Fc and dIgA apically and basolaterally, respectively. To study dynamics of FcRn-dependent traffic in real time, we will use a high speed synchronized spinning disk Confocal Imaging System (UltraVIEW ERS) with a sensitive EMCCD camera. To overcome bleaching problems associated with constant illumination during live imaging, bleach-resistant Alexa-dyes will be directly coupled to ligands; also an exceptionally photo-stable streptavidin-coated Qdot-565 will be used to label biotinylated human Fc. Biotinylated hFc will be incubated with Qdot-streptavidin to follow the fates of FcRn-Fc complexes in polarized epithelial cells.

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378. A freeze substitution fixation-based gold enlarging technique for EM studies of endocytosed nanogold-labeled molecules

Wanzhong He[1]*, Christine Kivork*[2]*, Suman Machinani*[3]*, Mary K. Morphew*[4]*, Anna M. Gail*[5]*, Devin B. Tesar, Noreen E. Tiangco, J.R. McIntosh*[4]

We have developed methods to locate individual ligands that can be used for electron microscopy studies of dynamic events during endocytosis and subsequent intracellular trafficking. The methods are based on enlargement of 1.4 nm Nanogold attached to an endocytosed ligand. Nanogold, a small label that does not induce misdirection of ligand-receptor complexes, is ideal for labeling ligands endocytosed by live cells, but is too small to be routinely located in cells by electron microscopy. Traditional pre-embedding enhancement protocols to enlarge Nanogold are not compatible with high-pressure freezing/freeze substitution fixation (HPF/FSF), the most accurate method to preserve ultrastructure and dynamic events during trafficking. We have developed an improved enhancement procedure for chemically fixed samples that reduced autonucleation, and a new pre-embedding gold-enlarging technique for HPF/FSF samples that preserved contrast and ultrastructure and can be used for high-resolution tomography. We evaluated our methods using labeled Fc as a ligand for the neonatal Fc receptor. Attachment of Nanogold to Fc did not interfere with receptor binding or uptake, and gold-labeled Fc could be specifically enlarged to allow identification in 2D projections and in tomograms. These methods should be broadly applicable to many endocytosis and transcytosis studies.

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379. Three-dimensional itinerary of FcRn-mediated antibody transport across epithelial cells revealed by electron tomography

Wanzhong He[1]*, Grant J. Jensen*[2]*, J. Richard McIntosh*[3]

Prior to development of a fully functional immune system, mammalian young depend on maternal IgG transported across epithelial barriers by the neonatal Fc receptor (FcRn). In newborn rodents, FcRn transfers IgG in milk from the apical side of intestinal epithelial cells to the basolateral surface, where it enters the blood. Here we used electron tomography to derive a 3D map of transport vesicles in neonatal intestinal epithelial cells at 4-6 nm resolution. Suckling rats were fed Fc labeled with 1.4nm Nanogold, which was enlarged in high-pressure

frozen/freeze substitution fixed intestinal samples to enable identification of individual FcRn ligands. Tomograms of epithelial cells revealed Fc in coated pits at the apical surface, inside tubular and irregular vesicles that form a tangled web associated with microtubules, in multivesicular bodies (MVBs), and in clathrin-coated vesicles and coated pits near and at the basolateral surface. The transcytosis pathways inferred from these snapshots of dynamic events include unanticipated features, such as association with MVB inner vesicles and MVB tubules and transit through pleomorphic clathrin-coated tubules and vesicles.

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380. Intracellular trafficking of an antibody bipolar bridged complex of HSV-1 Fc receptor-antiviral IgG

Alex Farley

Herpes Simplex Virus (HSV) is a model member of the alphaherpesvirus family, which also includes Varicella-Zoster Virus (ZVZ) and Pseudorabies Virus (PrV). Alphaherpesviruses are characterized by a relatively short replicative cycle in epithelial tissues and egression to and latent infection of the sensory neurons. HSV encodes two type 1 membrane-bound glycoproteins which together function as a receptor for the Fc portion of IgG. The heterodimer is composed of glycoprotein E (gE) and glycoprotein (gI) and it is found on the surface of virions and infected cells. The Fc receptor function of gE-gI is thought to provide a mechanism for immune evasion by blocking the effector function of host Fc recognition proteins in both the adaptive and innate immune systems. The gE-gI heterodimer is thought to bind to antigen-bound IgG in a process called bipolar bridging in which the antigen-binding fragments (Fabs) bind to an antigen, and gE-gI binds to the Fc. This process could provide a mechanism for HSV-1 to evade antibody-mediated immune responses via Fc receptors. The binding affinity of gE-gI for Fc is pH dependent, whereby gE-gI binds IgG with high affinity at the slightly basic pH of the cell surface (~7.4) and releases IgG at acidic pH (≤6.4). The sharply pH-dependent binding suggests that IgG that is endocytosed by gE-gI dissociates from gE-gI at the low pH of endosomal compartments, where it could be degraded, while gE-gI is recycled back to the cell surface. Our hypothesis is that Fc binding to gE-gI results in endocytosis of the antibody/antigen complex and its consequent degradation possibly in the lysosome. We have shown that the gE-gI heterodimer can internalize the Fc domain of IgG. Future work will include experiments to address the intracellular trafficking patterns of gE-gI and the fates of gE-gI/IgG and gE-gI/IgG/antigen complexes.

381. The human cytomegalovirus Fc receptor gp68 binds the Fc C_H2-C_H3 interface of IgG2

Elizabeth R. Sprague[1], Henrike Reinhard[2], Evelyn J. Cheung, Alex Farley, Robin Deis Trujillo[3], Hartmut Hengel[2]

Surface receptors for the Fc domain of immunoglobulin G (Fcγ), FcγRs, represent immune receptors connecting humoral and cellular immune responses. Two-human cytomegalovirus (HCMV)-encoded type I transmembrane receptors with Fcγ-binding properties (vFcγRs), gp34 and gp68, have been identified on the surface of HCMV-infected cells, and are assumed to confer protection against IgG-mediated immunity. Here we show that Fcγ recognition by both vFcγRs occurs independent of N-linked glycosylation of Fcγ, contrasting with the properties of host FcγRs. To gain further insight into the interaction with Fcγ, truncations of the vFcγR gp68 ectodomain were probed for Fcγ binding, resulting in localization of the Fcγ binding site on gp68 to residues 71 – 289, a region including an immunoglobulin-like domain. Gel filtration and biosensor binding experiments revealed that, unlike host FcγRs but similar to the Herpes simplex virus 1 (HSV-1) Fc receptor gE-gI, gp68 binds to the C_H2-C_H3 interdomain interface of the Fcγ dimer with a nanomolar affinity and a 2:1 stoichiometry. Unlike gE-gI, which binds Fcγ at the slightly basic pH of the extracellular milieu but not at the acidic pH of endosomes, the affinity of the gp68/Fcγ complex is approximately constant from pH values 5.6 to 8.1. These data indicate that the mechanistic details of Fc-binding by HCMV gp68 differ from host FcγRs and from HSV-1 gE-gI, suggesting distinct functional and recognition properties.

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382. Crystal structure of the peptide-binding viral class I MHC homolog, UL18, bound to LIR-1, a host and viral MHC receptor

Zhiru (Jenny) Yang

HCMV affects 70-90% of all human populations and can be life threatening to immunocompromised individuals, such as cancer, transplant, and AIDS patients. HCMV achieves its lifelong infection in host cells by adopting multiple mechanisms to evade the primed immune system, including down-regulation of host class I MHC molecules. Perhaps as a means of avoiding host immune responses triggered by low levels of class I MHC, HCMV encodes a class I homolog called UL18, which unlike other viral and host MHC homologs, associates with endogenous peptides resembling those binding to host class I MHC. Unlike classical class I molecules, however, UL18 is heavily glycosylated (13 potential N-linked glycosylation sites). The host cell receptor for UL18 is LIR-1 (ILT-4), an inhibitory receptor similar to KIR

family members, but expressed on B cells, monocytes, macrophages, dendritic cells and only a subset of natural killer cells. In addition to UL18, LIR-1 binds a broad range of host MHC class I molecules, but with an affinity that is over 1000 times reduced compared to its affinity for UL18. The structures of LIR-1 and a LIR-1/HLA-A2 complex have recently been solved in our laboratory. We have solved the structure of UL18 bound to LIR-1 at 2.2 Å resolution, and compared it to the structure of a LIR-1/HLA-A2 complex. Although UL18 and HLA-A2 are related by only ~20% sequence identity, the structures are remarkably similar, including the modes of association with peptide and with the host cell-derived light chain, β2-microglobulin. Although LIR-1 associates with the α3 and β2-microglobulin domains of both UL18 and HLA-A2 in a similar way, it shows increased contacts to the UL18 α3 domain, rationalizing the higher affinity of the LIR-1/UL18 interaction.

383. Structural studies of class C GPCRs

Rich Olson

I am employing structural techniques to investigate members of the class C family of G-protein coupled receptors (GPRCs). This diverse collection of small ligand-activated proteins is characterized by a large extracellular ligand-binding domain in addition to the canonical seven-transmembrane helical domain common to all GPCRs. Members of this family include mammalian V2R pheromone receptors, calcium-sensing receptors, $GABA_B$ receptors, metabotropic glutamate receptors, taste receptors, and fish odorant receptors.

I have been using a green-fluorescent protein (GFP) based system for screening the expression and solution behavior of detergent-solubilized and soluble ectodomain constructs of different representative class C GPCR genes. Initial screens are carried out in transiently transfected human embryonic kidney (HEK) cells and attractive candidates are then transitioned into a baculovirus expression system for scale-up. An in-line fluorescence detection system coupled to a size exclusion chromatography column allows initial characterization of the oligomeric state and expression levels of GPCR constructs without having to carry out an initial purification. This is particularly useful in the case of membrane proteins, where expression levels are low and detergents are necessary for isolation. My initial screen of over 40 genes has yielded several candidates that look promising for further structural studies. X-ray crystallography and single-molecule electron microscopy techniques will be employed to investigate the ligand-selectivity and activation mechanism of representative class C GPCRs.

384. Structural studies of the NK lysis receptor NKp30

Tal I. Arnon

Natural killer (NK) cells are an important subset of the innate immunity lymphocytes capable of destroying a wide range of cells via a relatively limited set of activating receptors. NKp30 is one of the major NK-activating receptors that is directly involved in the recognition and killing of tumor and pathogen-infected cells. In addition, NKp30 plays a direct role in modulating the survival and maturation of dendritic cells, thus suggesting a wider role for NK cells in the overall immune response. NKp30 is known to bind a wide rang of target cells, but the only NKp30 specific ligand identified so far is the human cytomegalovirus derived protein pp65, which specifically antagonizes the NKp30 receptor leading to inhibition of NK cytotoxicity.

On NK cells, NKp30 encodes for a 190 amino acid polypeptide belonging to the immunoglobulin superfamily. In addition, mRNA analysis of NKp30 revealed that another alternative spliced isoform is expressed, displaying a shorter extracellular domain. However, nothing is yet known about the expression pattern and physiological relevance of this isoform.

We would like to study the structural and functional characteristics of both NKp30 isoforms alone and in complex with the pp65 ligand. For this purpose, we expressed the extracellular domain of NKp30 as C-terminally X6-HIS tagged proteins in insect cells. Both isoforms were recognized by polyclonal NKp30 Abs and displayed a specific and differential binding to various tumor cell lines. In addition, preliminary analysis suggests that the shorter NKp30 isoform forms dimers thus, indicating a possible different mechanism of operation. Using these proteins we set up initial crystallization trials. Detailed understanding of the function and structure of the NKp30 receptor may provide better understanding of basic biology of NK cells and the molecular mechanisms that underline their response during different pathological situation.

385. Structural studies of the protochordate histocompatibility system

*Tal I. Arnon, Anthony W. De Tomaso**

Botryllus schlosseri is a colonial marine urochordate that serves as a convenient model for studying primitive allorecognition in invertebrates. During his lifespan, *B. schlosseri* is subjected to frequent tissue-to-tissue contact between neighboring colonies. Upon contact, the two colonies either fuse or reject each other in a blood-based inflammatory reaction determined by a single highly polymorphic locus, named fusion histocompatibility complex (FuHC). This ability to distinguish between self and non-self have long been suggested to represent a possible evolutionary ancestor to the adaptive immune system and the development of the histocompatibility complex (MHC I, II) molecules in vertebrates. Recently, the FuHC gene locus in *B. schlosseri* was identified. FuHC is predicted to encode for a type I transmembrane protein with two extracellular immunoglobulin domains, followed by a transmembrane region and an intracellular tail with no known motives. In addition, an alternative spliced variant of FuHC also exists, predicted to encode for a shorter secreted protein. Although the molecular mechanisms that underline the fusion/rejection response are still unknown, a candidate ligand for the FuHC proteins was identified. This protein, named fester, was found in tight association with the FuHC gene and was demonstrated to play a direct role in

the fusion/rejection response. Fester is a highly polymorphic type I transmembrane receptor that is also predicted to encode for secreted protein due to alternative splicing events. We would like to determine the 3D structure of FuHC and fester and to characterize the possible interactions between these molecules. Such information may lead to a better understanding of the evolutionary origin of allorecognition systems in higher organisms and, ultimately may help to uncover some of the mystery associated with the development of the adaptive immune system.

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386. Improved neutralizing antibodies against HIV

Anthony P. West, Jr., Joshua Klein, Priyanthi Gnanapragasam, Chris Foglesong, Rachel Galimidi, Noreen Tiangco, Maria Suzuki, Lili Yang[1]*, David Baltimore*[2]

Together with David Baltimore's laboratory, we are attempting to develop a new "Engineering Immunity" approach to treating HIV/AIDS. This strategy envisions using lentiviral vectors to program a patient's immune system to produce designed anti-HIV molecules. The Bjorkman lab component of this project entails developing an improved set of antibody reagents that neutralize HIV. Although the vast majority of natural anti-HIV antibodies are highly strain-specific, a small number of broadly neutralizing anti-HIV antibodies have been identified. We are attempting to take these antibodies as a starting point to develop antibodies or antibody-like proteins engineered to bind more tightly to HIV or to recruit immune effector cells

One set of molecules we are investigating are CD4/CD4i Ab reagents. When gp120 binds CD4, a conformational change occurs exposing the coreceptor binding site. Antibodies targeting this CD4-induced site (so called CD4i Abs) often are broadly cross-reactive. Normally, they have little neutralization potency *in vivo* due to limited steric accessibility when gp120 is bound to cell surface CD4. Several years ago, to overcome this problem, Ed Berger's laboratory designed a bispecific reagent containing part of CD4 linked to the scFv form of 17b, a CD4i Ab. We would like to fully explore the potential of CD4/CD4i reagents since they target two conserved functional sites on gp120. These reagents have several components: domains 1 and 2 from CD4, a CD4i Ab combining site (scFv or Fab), linkers, and potentially an Fc domain to provide bivalency and an increased serum half-life. We are exploring different ways of combining these components, as well as comparing the effectiveness of several CD4i Abs. We hope to determine the reagent(s) with the most optimal combination of neutralization potency, expression efficiency, and half-life.

During purification of the neutralizing Ab 2G12, we observed that a small fraction of this Ab exists in a dimeric form. Our *in vitro* neutralization experiments demonstrate that this dimeric form of 2G12 is 20 to 30-fold more potent than the monomer. Based on a structural hypothesis on the nature of the dimer, we are attempting to design a mutated form of 2G12 that would yield a higher fraction of the dimer.

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387. Structural studies of novel HIV neutralizing epitopes

Anthony P. West, Jr., Noreen Tiangco, Rachel Galimidi, Chris Foglesong, Roland Strong[1]*, Leo Stamatatos*[2]

In collaboration with the Stamatatos and Strong Labs, we have begun a project to discover novel HIV neutralizing epitopes, which will then be developed through computational design for optimal presentation as small protein immunogens. Our lab will focus on determining the crystal structures of novel antibodies bound to gp120 or gp160. In preparation for these studies, we have isolated the cDNAs encoding two relatively new CD4i antibodies, 19e and 21c, and have expressed these in IgG and scFv forms. We have begun expression experiments on gp120s from several clade B and clade C strains, and started preliminary crystallization screening.

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388. Steric occlusion limits the potency of the broadly neutralizing HIV-1 antibody 4E10

Joshua S. Klein, Priyanthi Gnanapragasam, Rachel Galimidi, Noreen Tiangco, Chris Foglesong, Anthony P. West

Monoclonal antibody 4E10 is the most broadly neutralizing anti-HIV antibody characterized to date. Its epitope has been identified as a highly conserved linear sequence in the membrane proximal external region of gp41. Despite intense efforts to design immunogens capable of eliciting a broadly neutralizing gp41-specific response, none have met with success, and 4E10-like responses are exceedingly rare in HIV-infected individuals. One hypothesis put forward to explain the lack of 4E10-like responses to HIV is that 4E10 binds self-antigens such as phospholipids commonly present in cellular membranes, so B cells with 4E10-like receptors are deleted or anergized. Here we present evidence that the 4E10 epitope exists within a sterically constrained environment that limits access to the epitope by IgG 4E10 but is less restrictive to a Fab or smaller single chain Fv (scFv) derived from the same antibody. We observed IgG 4E10 to bind immobilized monomeric gp41 ectodomain with an apparent affinity that was approximately two orders of magnitude stronger than either Fab 4E10 or scFv 4E10. However, in an *in vitro* neutralization assay with a panel of eight pseudotyped primary HIV isolates, we observed that scFv 4E10 was, on average, only 3-fold less potent than IgG 4E10 on a molar basis, whereas Fab 4E10 was 7-fold less potent. In contrast, a scFv derived from b12, which is unlikely to suffer from any steric restriction because its epitope on gp120 probably faces out into the extracellular environment, was observed to be 16-fold less potent than its IgG format, whereas Fab b12 was observed to be 12-fold less potent. Our data suggest that even if B cells expressing 4E10-like B cell receptors are not deleted,

they still will not be activated to undergo clonal expansion because 4E10-like antibodies are sterically prohibited from accessing the 4E10 epitope on gp41 in its native membrane-bound state.

389. Crystallographic studies of the ternary complex of transferrin receptor with transferrin and HFE

Kathryn E. Huey-Tubman, Fan Yang, Anne B. Mason[*]

Iron is transported in the serum of vertebrates bound to transferrin (Tf). Transferrin receptor (TfR) assists iron uptake into vertebrate cells through a cycle of endo- and exocytosis of Tf. TfR binds iron-loaded Tf (Fe-Tf) at the cell surface (pH 7.4) and carries it to the endosome. Iron dissociates from Tf at the acidic pH of the endosome (pH 6.0), but Apo-Tf remains tightly bound to TfR. The Apo-Tf/TfR complex then returns to the cell surface and at extracellular pH, Apo-Tf dissociates and is replaced by diferric Tf from the serum. TfR also interacts with HFE, an MHC class I-related protein that is mutated in patients with the iron overload disorder, hereditary hemochromatosis. HFE binds to TfR, decreasing its ability to bind iron-loaded Tf. Previous crystallographic studies have revealed the structures of the ectodomain of TfR alone (*Lawrence et al.*, 1999) and in complex with HFE (*Bennett et al.*, 2000), as well as several Tf forms and family members. A low-resolution (7.5 Å) structure of the ternary complex, TfR bound to HFE and Tf, has been produced from cryoelectron microscopy (*Cheng et al.*, 2004). To further understand the mechanisms of TfR-facilitated iron release from Tf and how HFE affects iron uptake, we want to solve the structure of the Fe-Tf/TfR/HFE ternary complex. We have designed and expressed in insect cells a heterodimeric mutant of TfR where one chain of the dimer binds Tf and the other chain HFE. This along with various forms of wild-type and mutant iron-loaded and iron-free Tfs (expressed both in insect cells and BHK cells) and HFE (expressed in CHO cells) will allow us to set up numerous crystallization trials.

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References

Lawrence *et al*., *Science* (1999) **286**:779-782.
Bennett *et al*., *Nature* (2000) **403**:46-53.
Cheng *et al*., *Cell* (2004) **116**:565-576.

390. Understanding the interactions between the iron-regulatory protein hemojuvelin and its receptor neogenin

Fan Yang, Anthony P. West, Jr., An-Sheng Zhang[*]*, Caroline A. Enns*[*]

Hemojuvelin, a GPI-linker protein, is known to play an important role in maintaining iron homeostasis in mammals. Loss of function in this protein causes irreversible iron overload in vital organs, resulting in severe damage. Several studies have shown that hemojuvelin is the upstream regulator of hepcidin, the key iron-regulatory hormone secreted by hepatocytes. Several proteins have been suggested to be involved in this regulatory path, including neogenin, a transmembrane immunoglobulin gene superfamily receptor, which binds directly to hemojuvelin. We expressed soluble hemojuvelin and different versions of neogenin in an insect cell/baculovirus system. We demonstrated that the 6th fibronectin type III (FNIII) domain of neogenin contains the major hemojuvelin-binding epitope, with a minor contribution from the FNIII 5th domain and the ectodomain tail. Interestingly, a recombinant protein comprising the FNIII 5th and 6th domains plus the ectodomain tail showed more than 1000-fold higher affinity to hemojuvelin than the whole neogenin ectodomain when measured by a surface plasmon resonance-based binding assay, indicating a binding orientation preference suggesting mechanistic significance. Crystals have been obtained for the hemojuvelin-binding fragment alone, and attempts to crystallize the complex with hemojuvelin are underway.

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391. Biochemical and structural studies of ferroportin

Adrian E. Rice, Douglas C. Rees[*]

All known organisms, save two bacteria, require iron for survival. Despite its importance, iron in overabundance is toxic. In order to maintain a balance of iron levels, organisms have developed a highly specialized network of molecules designed to monitor and maintain iron homeostasis. When the fidelity of these networks is compromised, diseases such as iron deficiency and iron overload result. Mammals lack any regulated mode of iron excretion and therefore must have highly regulated mechanisms for controlling the acquisition of iron from the diet. The primary site of iron absorption is the duodenum of the small intestine. This process can be categorized by two phases: 1) iron uptake across the brush border into the cytoplasm of duodenal enterocytes; and 2) iron export across the basolateral membrane of these cells into the blood. Iron from the diet is reduced from Fe3+ to Fe2+ by the membrane-bound iron reductase Dcytb and is transported across the apical brush border by an integral membrane protein called divalent metal transporter 1 (DMT1; also known as DCT1 and NRAMP2). Iron is then transported across the cell to the basolateral side where it is exported by the basolateral integral membrane iron transporter ferroportin (Fpn; also known as IREG1 and MTP1). Fpn is the only identified iron exporter in vertebrates and is an integral membrane protein containing 9-12 predicted alpha-helical transmembrane segments. Point mutations in Fpn lead to an autosomal dominant iron overload disease called ferroportin disease. My project aims to characterize Fpn from both a structural and biochemical standpoint. Current plans in our laboratory aim at obtaining purified recombinant Fpn for these studies.

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392. Structural study of L1 homophilic adhesion by cryoelectron tomography

Yongning He, Grant Jensen[*]

L1 (CD171) is a cell surface molecule expressed on many cell types, especially on nerve tissues, and is involved in many cellular processes such as cell migration, axonal growth, and myelination. L1 mediates both homophilic and heterophilic adhesion in cells. L1-mediated homophilic adhesion is important for cell migration for both neural and tumor cells, which plays an important role in tumor invasion and migration. L1 is a transmembrane protein with a large extracellular portion, a single transmembrane domain and a cytoplasm tail. The extracellular region contains six immunoglobulin (Ig)-like domains and five fibronectin type III (FN) domains. Based on known structures of L1 homologues, the four N-terminal Ig-like domains are predicted to adopt a horseshoe-shaped conformation in which the first and the second Ig-like domain fold back to interact with the fourth and the third Ig-like domain, respectively. The role of the FN domains in adhesion remains unclear.

Cryo-electron tomography (cryoET) is a method to generate three-dimensional (3D) images of large macromolecular structures and complexes at around 3-4 nm resolution. Here we investigate L1-mediated homophilic adhesion in 3D using cryoET. Instead of using real cells for aggregation, we attached L1 ectodomains to liposomes to make a model system which functions in homophilic adhesion. In our tomograms, L1 homophilic adhesion shows recognizable features at the interfaces between adhering liposomes. The distance between adjacent membranes can be determined. Furthermore, we observe regular structures between neighboring L1 molecules at the adhesive interface. Based on the observed structure features, a potential homophilic adhesion model for L1 has been proposed.

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393. The structure of a polyQ–anti-polyQ complex reveals binding according to a linear lattice model

Pingwei Li[*], *Kathryn E. Huey-Tubman, Tiyu Gao*[*], *Xiaojun Li*[*], *Anthony P. West, Jr., Melanie J. Bennett*

Huntington and related neurological diseases result from expansion of a polyglutamine (polyQ) tract. The linear lattice model for the structure and binding properties of polyQ proposes that both expanded and normal polyQ tracts in the preaggregation state are random-coil structures but that an expanded polyQ repeat contains a larger number of epitopes recognized by antibodies or other proteins. The crystal structure of polyQ bound to MW1, an antibody against polyQ, reveals that polyQ adopts an extended, coil-like structure. Consistent with the linear lattice model, multimeric MW1 Fvs bind more tightly to longer than to shorter polyQ tracts and, compared with monomeric Fv, bind expanded polyQ repeats with higher apparent affinities. These results suggest a mechanism for the toxicity of expanded polyQ and a strategy to link anti-polyQ compounds to create high-avidity therapeutics.

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Publications

Bjorkman, P.J. (2006) Finding the groove. *Nature Immunology* **7**:787-789.

Delker, S.L., West, A.P., Jr., McDermott, L., Kennedy, M.W. and Bjorkman, P.J. (2004) Crystallographic studies of ligand binding by Zn-a2-glycoprotein. *J. Struct. Biol.* **148**:205-213.

Hamburger, A.E., Bjorkman, P.J. and Herr, A.B. (2006) Structural insights into antibody-mediated mucosal immunity. *Curr. Topics Microbiol. & Immmunol.* **308**:173-204.

Hamburger, A.E., West, A.P., Jr. and Bjorkman, P.J. (2004) Crystal structure of a polymeric immunoglobulin-binding fragment of the human polymeric immunoglobulin receptor. *Structure* **12**:1925-1935.

He, W., Jensen, G.J., McIntosh, J.R. and Bjorkman, P.J. (2007) Three-dimensional itinerary of FcRn-mediated antibody transport across epithelial cells revealed by electron tomography. Manuscript submitted.

He, W., Kivork, C., Machinani, S. Morphew, M.K., Gail, A.M., Tesar, D.B., Tiangco, N.E., McIntosh, J.M. and Bjorkman, P.J. (2007) A freeze substitution fixation-based gold enlarging technique for EM studies of endocytosed nanogold-labeled molecules. *J. Struct. Biol.* In press.

Ja, W.W., West, A.P. Jr., Delker, S.L., Bjorkman, P.J., Benzer, S. and Roberts, R.W. (2007) Extension of *Drosophila* lifespan with a G protein-coupled receptor inhibitor. *Nature Chem. Biol.* In press.

Kacskovics, I., Kis, Z., Mayer, B., West, A.P., Tiangco, N.E., Tilahun, M., Cervenak, L., Bjorkman, P.J., Goldsby, R.A., Szenci, O. and Hammarström, L. (2006) FcRn mediates elongated serum half-life of human IgG in the cattle. *Internat. Immunol.* **18**:525-536.

Li, P., Huey Tubman, K.E., Gao, T., Li, X., West, A.P. Jr., Bennett, M.J. and Bjorkman, P.J. (2007) The structure of a poly-Q/anti-poly-Q complex reveals binding according to a linear lattice model. *Nature Struct. & Molec. Biol.* **14**:381-387.

Morphew, M., He, W., Bjorkman, P.J. and McIntosh, J.R. (2007) Silver enhancement of nanogold particles during freeze substitution fixation for electron microscopy. *J. Microscopy.* In press.

Olson, R., Dulac, C. and Bjorkman, P.J. (2006) MHC homologs in the nervous system - They haven't lost their groove. *Curr. Opin. Neurobiol.* **16**:351-357.

Sprague, E.R., Reinhard, H., Cheung, E.J., Farley, A.H., Trujillo, R.D., Hengel, H. and Bjorkman, P.J. (2007) The human cytomegalovirus Fc receptor gp68 binds the Fc C_H2-C_H3 interface of IgG. Manuscript submitted.

Sprague, E.R., Wang, C., Baker, D. and Bjorkman, P.J. (2006) Crystal structure of the HSV-1 Fc receptor bound to Fc reveals a mechanism for antibody bipolar bridging. *Public Library of Science (PLoS): Biology* **4**:525-536.

Tesar, D.B., Tiangco, N.E. and Bjorkman, P.J. (2006) Ligand valency affects FcRn-mediated transcytosis, recycling, and intracellular trafficking. *Traffic* **7**:1-16.

Professor Emeritus: Charles J. Brokaw

Summary: Motor enzymes — dyneins, kinesins, and myosins — convert energy from ATP dephosphorylation into most of the movements performed by eukaryotic cells. We think that myosin and kinesin are reasonably well understood, although new experimental results from time to time surprise us. On the other hand, we have very little knowledge or understanding of the functioning of the axonemal dyneins that power the movements of flagella and cilia; these molecular complexes are a major challenge for the future. My current work uses computer simulation methods to explore ideas about motor enzyme function in situations ranging from experimental studies on individual motors to an intact flagellum containing tens of thousands of dyneins. Some of the simulation programs, as Macintosh applications, are available at: www.cco.caltech.edu/~brokawc/software.html

394. The sliding initiation event in flagellar oscillation

Charles J. Brokaw

On flagella generating planar bending waves, there are two types of bends: basal bends and propagating bends. Basal bends are formed by interdoublet sliding distal to the bend. Sliding within the bend, in the opposite direction, transforms the basal bend into a propagating bend, and initiates development of a new basal bend. The initiation of this sliding is the sliding initiation event. The frequency of flagellar beating is the frequency of sliding initiation events (in one direction).

In the sliding initiation event, sliding appears to be initiated synchronously and uniformly throughout the basal bend, sometime after the cessation of sliding in the opposite direction that occurred during bend formation. It has been suggested that this represents a sudden activation of dynein motor enzymes throughout the bend. This synchronous activation of dyneins was originally suggested to explain the synchronous activation of sliding in a straight cilium at the beginning of its effective stroke. However, no known mechanism can explain this synchronous activation.

New image analysis methods are being applied to examine old photographs of flagellar bending. These provide a detailed record of sliding in the basal regions of demembranated spermatozoa, swimming in reactivation solutions containing ATP. Additional data is obtained from flagella attached to a slide surface, with only the distal 10 to 15 μm free to beat. These show highly asymmetric basal bending at frequencies near 1/sec in reactivation solutions containing only 10 μM ATP. This is a simpler situation in which external viscous resistance and the effects of bending beyond the basal region are largely eliminated. The sliding initiation events in these examples can be reproduced closely by computer simulations in which dyneins are switched off and on by curvature of the flagellum, with the dyneins on one side being turned on locally at the same time that the dyneins on the other side are turned off. This switching occurs locally as a bend grows and propagates, as in previous models using curvature-controlled switching. This agreement between models and data is highly dependent on the choices of kinetic parameters for the dynein motors and on the choice of the difference in kinetics between on and off states of dyneins. Nevertheless, it leads to the conclusion that data from simple sperm flagella do not demonstrate any need for a mechanism that activates dyneins synchronously throughout a length of the flagellum.

Professor of Chemistry and Biology: Judith L. Campbell
Member of the Professional Staff: Elizabeth Bertani, Martin Budd, Piotr Polaczek
Postdoctoral Scholars: Hui-Qiang Lou, Taro Masuda-Sasa
Graduate Students: Barbara Kraatz Fortini
Laboratory Staff: Santiago Laparra

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Summary: A hallmark of cancer cells, in addition to uncontrolled proliferation, is genomic instability, which appears in the form of chromosome loss or gain, gross chromosomal rearrangements, deletions, or amplifications. The mechanisms that suppress such instability are of the utmost interest in understanding the pathogenesis of cancer. Our lab studies the components of the DNA replication apparatus that promote genomic stability, primarily using yeast genetics, biochemistry, and functional genomics.

Several years ago, Rajiv Dua in the laboratory discovered that DNA polymerase ε, one of four essential DNA polymerases in yeast, had not one, but two essential functions. Deletion of the polymerase domain left the cells viable because another polymerase activity could substitute. Conversely, deletion of the remaining, non-catalytic half of the protein was lethal. Shaune Edwards in the laboratory carried out a two-hybrid screen for proteins that interact with the enigmatic C-terminal region of pol ε in order to discover its function. She found that pol ε interacts with Trf5, a protein involved in establishing cohesion of sister chromatids during passage of the replication fork. She has gone on to develop evidence that the essential function of the C terminus of pol ε is to aid in establishing efficient sister chromatid cohesion during S phase. Another postdoctoral fellow in the laboratory, Caroline Li has characterized the Trf5 protein. She has shown that it encodes a previously unknown poly A polymerase and that it stimulates the activity of pol ε dramatically. Future studies are aimed at defining the mechanism by which these two proteins regulate interaction of the replisome with the cohesin complex, the glue that holds the chromosomes together, and how failure of cohesion leads to genomic instability.

At least seven human diseases characterized by cancer predisposition and/or premature aging are correlated with defects in genes encoding DNA helicases. The yeast genome contains 134 open reading frames with helicase motifs, only eight of which have been characterized. Martin Budd in our laboratory identified the first eukaryotic helicase essential for DNA replication, Dna2. He showed by interaction studies that it was a component of the machine that is required for accurate processing of Okazaki fragments during lagging strand DNA replication. Enzymatic studies to elucidate the sequential action of the DNA polymerase, helicase, and nuclease required for this processing form an ongoing mechanistic biochemistry project in the laboratory.

Stimulated by various reports in the literature implicating Dna2 in telomere biogenesis and structure, Wonchae Choe made the interesting observation that the bulk of Dna2 is localized to telomeres and that this localization is dynamic. During G1 and G2 phases of the cell cycle, Dna2 is at telomeres. During S phase Dna2 is present on the replicating chromatin. Current studies are aimed at defining the genes that regulate the localization, including phosphorylation by the yeast ATR ortholog, Mec1. In addition to defects in replication, *dna2* mutants are also very sensitive to agents that induce double strand breaks (DSBs). Osamu Imamura has shown that Dna2 is mobilized from telomeres in response to the induction of double strand breaks. He is carrying out experiments to test the model that Dna2 delocalization from telomeres is part of the signaling system that induces the DNA damage and S phase checkpoints, as has also been suggested for yKU, a protein involved in non-homologous end joining and in stabilizing telomeres.

One model of cellular aging suggests that accumulation of DNA damage leads to replicative senescence. Most endogenous damage occurs during S phase and leads to replication fork stress. At least three human diseases of premature aging or cancer predisposition, Werner, Bloom, and Rothmund-Thompson, are caused by defects in helicases similar to Dna2. Martin Budd and Laura Hoopes found that *dna2* mutants have a significantly reduced life span. Microarray analysis by Isabelle Lesur shows that the *dna2* mutants age by the same pathway as wild-type cells; they just age faster. Interestingly, the human Bloom and Werner genes suppress the replication defect of *dna2* mutants. Yeast transcriptome analysis shows that old *dna2* mutants have a gene expression pattern strikingly similar to cells senescing due to telomerase defects. Future work will take advantage of the yeast system to further delineate the role of BLM and WRN proteins in mammalian cells. The work of Tao Wei in the lab suggests that instability of repetitive DNA, such as the ribosomal locus and telomeric DNA, is a major cause of genomic instability in the aging *dna2* mutants.

395. Multiple nucleases and helicases participate in telomere maintenance and DSB repair

Martin Budd, Taro Masuda-Sasa, Judith L. Campbell

Overlapping functions and participation in multiple pathways in DNA repair and genome stability have made it difficult to determine the distinct mechanisms by which many specific enzymes contribute. We have been trying to sort out some of these mechanisms. Dna2 is an essential 5'->3' helicase and nuclease involved in Okazaki fragment processing. Dna2 associates with telomeres during G1, then associates with chromosomal DNA during S phase, then reassociates with telomeres

during G2. *De novo* telomere synthesis is defective in *dna2-2* mutants. Pif1 is a 5'->3' helicase involved in mitochondrial DNA recombination, chromosomal DNA replication, and inhibition of telomere synthesis. *pif1Δ* mutants have long telomeres. Mre11 is a 3'-> 5' nuclease that is in a complex with Rad50 and Xrs2. Mre11 creates the 3' G-rich overhang at telomeres and is the major player in 5'-> 3'exonuclease processing at chromosomal breaks. *mre11Δ* strains have short telomeres and are extremely sensitive to x-rays. *pif1Δ* suppresses the lethality of a *dna2Δ* mutation. The strain is temperature sensitive, sensitive to MMS, and has synthetic lethal interactions similar to *dna2-1* and *dna2-2* mutants, such as synthetic lethality with *mre11Δ* mutants. Surprisingly, however, *dna2Δ pif1Δ*, unlike *dna2-2*, is resistant to x-rays. Thus, Pif1 performs some interfering function at DSBs in the absence of Dna2.

mre11Δ strains complemented with nuclease minus Mre11 proteins, Mre11-D56N or Mre11-H125N, are x-ray resistant and possess telomeres with WT length. Therefore, although Mre11 is required, the nuclease activity of Mre11 is not required for its function in processing either chromosomal breaks or telomere ends, suggesting other nucleases are involved. We have found that Dna2 may provide the nuclease function in the absence of Mre11 nuclease. We have constructed an *mre11D dna2Dpif1Δ* strain complemented with pMRE11, mutant pMre11-D56N and pMre11-H125N plasmids. The *mre11Δ dna2Δ pif1Δ* (pMRE11) strain is resistant to x-rays. However, both the *mre11Δ dna2Δ pif1Δ* (pMre11-D56N) and *mre11Δ dna2Δ pif1Δ* (pMre11-H125N) strains are sensitive to x-rays.

In addition to being involved in telomere and DSB processing, another possible role for Dna2 at telomeres is processing of Okazaki fragments either during conventional replication of telomeric repeats or during resynthesis of the C-rich strand after telomerase extension. We have found that temperature sensitive *dna2-2 exo1Δ* strains have extensive single stranded G-rich telomere DNA at 37^{o}C. The same phenotype has been demonstrated by others for *rad27Δ*, lacking FEN1 nuclease. We conclude that FEN1 is not sufficient for Okazaki fragment processing at telomeres and that either Exo1, a member of the FEN1 family, or Dna2 is required in addition.

Finally, we have also found that Dna2 nuclease suppresses the creation of potentially toxic substrates created by Dna2 helicase, suggesting they are coordinated activities. We will present genetic evidence supporting this.

396. Mrc1: A possible coordinator at replication forks

Huiqiang Lou, Judith L. Campbell

Complicated replication machines are sequentially assembled and precisely programmed during the M/G1 phases of the cell cycle. How to prevent replication forks from irreversibly collapsing in S phase, when *de novo* assembly and programming are no longer permitted, becomes a fascinating question.

As one of the essential DNA polymerases, the only indispensable function of Pol epsilon lies at the extreme C-terminus of Pol2, which forms the scaffold platform of replication machinery. It's feasible that this domain might be important for fork stabilization through association with other components. Our finding of the *in vivo* interaction between Mrc1 and Pol2 might shed light on this mechanism. Yeast Mrc1 is a mediator in the S phase checkpoint pathway. It is also required for the proper assembly of the replication fork at origins (similar timing as Pol ε) and for normal fork progression. But how does Mrc1 work at replication forks?

Mrc1 partially coimmunoprecipitates with Pol2, while almost all Pol2 molecules precipitate with Mrc1 in asynchronized cells. This suggests that Pol2 associates with Mrc1 throughout the whole cell cycle, which is further confirmed by using α-factor arrested (G1) and HU-arrested (S) cells. HU-treated cells also show that both phosphorylated and nonphosphorylated Mrc1 bind to Pol2 with similar efficiency. The interaction remains undisturbed with up to 250 mM salt. More strikingly, Mrc1 interacts with both the functional portion of Pol 2 (Pol2N catalytic domain) and the structural domain (Pol2C) independently, and that the former interaction is abolished by phosphorylation of Mrc1 in the S phase checkpoint signaling pathway. Through a set of N-or C-terminal truncation mutants of Mrc1, N-terminus (140-450aa) of Mrc1 seems to be responsible for binding with Pol2N, while the conserved C-terminus region (650-890aa) seems to be required for association with pol2C. We also characterized the interactions functionally using yeast genetic techniques - synthetic lethality and suppression.

397. Dna2 helicase/nuclease is a substrate of Mec1

Barbara Kraatz Fortini

The Dna2 protein is a yeast helicase/nuclease that is involved in Okazaki fragment processing, repair of x-ray and MMS induced DNA damage, rDNA stability, and the telomere position effect. We have shown previously that Dna2 exhibits an unusual subcellular distribution for a replication protein. The bulk of Dna2 is localized to telomeres in G1, leaves telomeres in S phase to be found at internal chromosomal sites, and returns to telomeres in G2. In addition, upon treatment with DNA damaging agents, Dna2 is released from telomeres. We have now shown that damage-induced release from telomeres depends on the Mec1 checkpoint kinase. Further, we utilized an *in vitro* kinase assay to demonstrate that Dna2 is an efficient substrate of purified Mec1 kinase. Mec1 is member of the PI-3-kinase-related kinase (PIKK) family whose members preferentially phosphorylate SQ or TQ motifs upon DNA damage. To ascertain whether Dna2 is an *in vivo* substrate of Mec1, we used site-directed mutagenesis to alter the putative Mec1 phosphorylation sites in Dna2 to alanine residues and analyzed the phenotypes of the resulting mutants. Two of these point mutants show increased DNA

damage sensitivity to multiple types of DNA damage including double strand breaks and single strand lesions, suggesting Mec1 phosphorylation plays a role in regulating the *in vivo* activity of Dna2. Current efforts involve large-scale purification of Dna2 mutant proteins using FPLC and affinity chromatography for use in enzymatic activity assays. These mutants are tested for *in vitro* ATPase, nuclease, and helicase activity, and this data used in conjunction with the *in vivo* phenotypes of the mutants. Comparing enzymatic activity to cellular activity will show us what roles regulation by other proteins play in regulating the activity of the Dna2 enzyme in cells after DNA damage.

398. G-quartet processing activity of DNA2

Taro Masuda-Sasa, Lu Chen, Piotr J. Polaczek

DNA2 is an essential gene conserved from yeast to human. Budding yeast Dna2p (ScDna2p) is a nuclease/helicase assisting FEN1 nuclease in processing a subset of Okazaki fragments that have long, single-stranded 5' flaps. Additionally, *DNA2* performs an essential function at telomeres. In *S. cerevisiae*, *dna2* mutants have defects in the maintenance of telomeres and in *de novo* telomere synthesis, and ScDna2p shows dynamic localization to telomeres in G1 and G2 phases. However, the exact function of Dna2p at telomeres is not clear. Various stable structures, referred to collectively as G-quartets, form readily from DNA fragments containing runs of guanines that are found at the rDNA (the *RDN1* locus) and telomeres. G-quartet formation is based on interactions between G residues that can form square-planar arrays of four G's associated through Hoogsteen base pairing. Since G-quartets are generally resistant to various enzymes such as nucleases and helicases and are inhibitory to telomerase, formation of G-quartets is thought to interfere with telomere elongation and DNA replication. We examined the interaction between Dna2p and G-quartet structures to investigate the possibility that Dna2p removes these structures. We found that oligonucleotides with yeast telomeric repeats of $(TGTGTGGG)_3$, flanked by single-stranded sequence on each end, formed a structure in the presence of Na^+ with the electrophoretic mobility expected for four-stranded intermolecular G-quartet DNA. HsDna2 and ScDna2 show higher affinity for G-quartet structures than for single-stranded oligonucleotides. Moreover, we demonstrated that HsDna2 and ScDna2 helicase can resolve G-quartets into single strands. Dna2 showed similar activity against other, well-characterized intermolecular G-quartet structures, such as the *Oxitricha* telomeric sequence. Finally, an intramolecular human telomeric G-quartet DNA structure is generally resistant to the nuclease cleavage. However, Dna2p in the presence of RPA can efficiently cleave such structures. Taken together, these results suggest an important role for Dna2 in resolution of intermolecular and intramolecular G-quartet structures.

Publications

Boronat, S. and Campbell, J.L. (2007) Mitotic Cdc6 stabilizes anaphase-promoting complex substrates by a partially Cdc28-independent mechanism, and this stabilization is suppressed by deletion of Cdc55. *Mol. Cell. Biol.* **27**:1158-1171.

Budd, M.E., Reis, C.C., Smith, S., Myung, K. and Campbell, J.L. (2006) Evidence suggesting that Pif1 helicase functions in DNA replication with the DNA2 helicase/nuclease and DNA polymerase delta. *Mol. Cell. Biol.* **26**(7):2490-2500.

Campbell, J.L. and Modrich, P. (eds.) (2006) Methods Enzymol. DNA Repair, Part A, vol. 324, Elsevier, New York.

Campbell, J.L. and Modrich, P. (eds.) (2006) Methods Enzymol. DNA Repair, Part A, vol. 324B, Elsevier, New York.

Masuda-Sasa, T., Imamura, O. and Campbell, J.L. (2006) Biochemical analysis of human Dna2. *Nucl. Acids Res.* **34**:1865-1875.

Masuda-Sasa, T., Polaczek, P. and Campbell, J.L. (2007) Single-strand annealing and ATP-independent strand exchange activities of yeast and human DNA2: possible role in Okazki fragment maturation. *J. Biol. Chem.* **281**:38555-64.

Reis, C. and Campbell, J. L. (2007) Contribution of Trf4/5 and the nuclear exosome to genome stability through regulation of histone mRNA levels in *Saccharomyces cerevisiae*. *Genetics* **175**:993-1010.

Stewart, J., Campbell, J.L. and Bambara, R. (2007) Flap endonuclease disengages DNA2 helicase/nuclease from Okazaki fragment flaps. *J. Biol. Chem.* **281**:38565-38572.

Stewart, J.A., Campbell, J.L. and Bambara, R.A. (2006) Flap endonuclease disengages Dna2 helicase/nuclease from Okazaki fragment flaps. *J. Biol. Chem.* **281**:38565-38572.

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Summary: The primary focus of our lab is to understand the role of mitochondrial dynamics in normal cellular function and human disease. Mitochondria are remarkably dynamic organelles that undergo continual cycles of fusion and fission. The equilibrium of these two opposing processes determines the overall morphology of mitochondria in cells and has important consequences for mitochondrial function.

Our research falls into several broad areas:

(1) What are the cellular and physiological functions of mitochondrial fusion and fission?
(2) What is the molecular mechanism of mitochondrial membrane fusion and fission?
(3) What role do mitochondrial dynamics play in human diseases?

To address these issues, we use a wide range of approaches, including genetics, biochemistry, cell biology, and structural biology.

Cellular and physiological functions of mitochondrial fusion and fission

A typical mammalian cell can have hundreds of mitochondria. However, each mitochondrion is not autonomous, because fusion and fission events mix mitochondrial membranes and contents. As a result, such events have major implications for the function of the mitochondrial population. We are interested in understanding the cellular role of mitochondrial dynamics, and how changes in mitochondrial dynamics can affect the function of vertebrate tissues. Recent findings indicate that perturbations in mitochondrial dynamics lead to neurodegeneration.

Much of our work focuses on proteins called mitofusins (Mfn1 and Mfn2), which are transmembrane GTPases embedded in the outer membrane of mitochondria. These proteins are essential for fusion of mitochondria. To understand the role of mitochondrial fusion in vertebrates, we have constructed mice deficient in either Mfn1 or Mfn2. We find that mice deficient in either Mfn1 or Mfn2 die in mid-gestation. Mfn2 mutant embryos have a specific and severe disruption of a layer of the placenta called the trophoblast giant cell layer. These findings indicate that mitochondrial fusion is essential for embryonic development due to a cell type-specific dependence on mitochondrial fusion.

Mfn2 is mutated in the human peripheral neuropathy Charcot-Marie-Tooth type 2A (CMT2A), in which long motor and sensory neurons are selectively affected. To understand the relationship between mitochondrial fusion and neurodegeneration, we have made conditional alleles of Mfn1 and Mfn2 in mice (Hsiuchen Chen). Analysis of these mice indicates that loss of Mfn2 leads to neurodegeneration in Purkinje neurons in the cerebellum. These studies clarify why mitochondrial dynamics is important for neuronal function and provide clue to the pathogenesis of CMT2A. We have also developed other mouse models, including transgenic and knock-in lines, to understand CMT2A (Scott Detmer).

Embryonic fibroblasts lacking Mfn1 or Mfn2 display fragmented mitochondria, a phenotype due to a severe reduction in mitochondrial fusion. Cells lacking both Mfn1 and Mfn2 have completely fragmented mitochondria and show no detectable mitochondrial fusion activity. Our analysis indicates that mitochondrial fusion is important not only for maintenance of mitochondrial morphology, but also for cell growth, mitochondrial membrane potential, and respiration. Our results indicate that dynamics, by enabling content exchange between mitochondria, provides a pathway to maintain essential components within mitochondria (Hsiuchen Chen).

Molecular mechanism of membrane fusion and fission

The best understood membrane fusion proteins are viral envelope proteins and SNARE complexes. Viral envelope proteins, such as gp41 of HIV, reside on the lipid surface of viruses and mediate fusion between the viral and cellular membranes during virus entry. SNARE complexes mediate a wide range of membrane fusion events between cellular membranes. In both cases, cellular and crystallographic studies have shown that the formation of helical bundles plays a critical role in bringing the merging membrane together. We would like to understand mitochondrial fusion and fission at a similar level of resolution and to determine whether there are common features to these diverse forms of membrane trafficking.

We are using structural biology to understand the mechanisms of mitochondrial fusion and fission. We have recently solved the structure of a portion of the mitochondrial fission apparatus (Yan Zhang). A mechanistic understanding of fission is crucial, because recent evidence indicates that mitochondrial fission is an important component of apoptosis.

Mitochondrial dynamics in human disease

Two inherited human diseases are caused by defects in mitochondrial dynamics. Charcot-Marie-Tooth (CMT) disease is a neurological disorder that affects the peripheral nerves. Patients with CMT experience progressive weakness of the distal limbs and some loss of sensation. A specific type of CMT, termed CMT2A, is caused by mutations in Mfn2 and result from degeneration of axons in peripheral nerves. We are currently analyzing the

functional consequences of such disease alleles, and using transgenic and targeted mutagenesis approaches to develop mouse models (Scott Detmer and Hsiuchen Chen).

The most common inherited form of optic neuropathy (autosomal dominant optic atrophy) is caused by mutations in OPA1. This mitochondrial protein is localized to the intermembrane space and is essential for mitochondrial fusion. Little is known about the mechanism of OPA1 action. In particular, there has been no good cell culture system to perform basic structure-function analysis of OPA1. To develop such a system, we have collaborated with Christiane Alexander (Max-Delbrück-Center for Molecular Medicine) to isolate mouse cell lines lacking OPA1 (Hsiuchen Chen). Using these cell lines, we have clarified the role of OPA1 in mitochondrial fusion and have identified the i-AAA protease Yme1L as a regulator of OPA1 processing (Zhiyin Song). Ultimately, a clear understanding of OPA1 will come from biochemical analysis, and we have developed expression systems that will enable such studies (Tadato Ban).

Finally, an understanding of mitochondrial dynamics will be essential for understanding a large collection of diseases termed mitochondrial encephalomyopathies. Many mitochondrial encephalomyopathies result from mutations in mitochondrial DNA (mtDNA). In mtDNA diseases, tissues maintain their mitochondrial function until pathogenic mtDNA levels exceed a critical threshold. Experiments with cell hybrids indicate that mitochondrial fusion, by enabling cooperation between mitochondria, can protect respiration even when >50% of mtDNAs are mutant. To understand the pathogenesis of mtDNA diseases, it is critical to explore how mitochondria can be functionally distinct and yet cooperate as a population within a cell. We anticipate that our studies with mice lacking mitochondrial fusion will help to shed light on this group of often devastating diseases (Hsiuchen Chen).

399. Mitochondrial fusion protects against cerebellar neurodegeneration

Hsiuchen Chen

Mitochondrial fusion is essential for normal development and physiology. Genetic targeting of each of the three known fusion genes, *Mfn1, Mfn2, and OPA1*, leads to embryonic lethality. The importance of mitochondrial fusion is further underscored by the fact that two neurodegenerative diseases, Charcot-Marie-Tooth disease type 2A (CMT) and autosomal dominant optic atrophy (DOA), are caused by mutations in *Mfn2* and *OPA1*, respectively.

To further explore the physiological requirements for mitochondrial fusion, we have created conditional knockout mice for both *Mfn1* and *Mfn2*. By targeting the embryo proper and leaving the placenta intact, we obtain live Mfn1-deficient pups and Mfn2-deficient pups. This indicates that the embryonic lethality associated with both knockouts is due to placental insufficiencies.

By removing *Mfn2* from the cerebellum, we established a model for neurodegeneration caused by loss of mitochondrial fusion. During development and after maturity, Purkinje cells require Mfn2 but not Mfn1 for dendritic outgrowth, spine formation, and cell survival. *In vivo*, cell culture, and electron microscopy studies indicate that mutant Purkinje cells have aberrant mitochondrial distribution, ultrastructure, and electron transport chain activity. In fibroblasts lacking mitochondrial fusion, the majority of mitochondria lack mitochondrial DNA nucleoids. This deficiency provides a molecular mechanism for the dependence of respiratory activity on mitochondrial fusion. Our results show that exchange of mitochondrial contents is important for mitochondrial function as well as organelle distribution in neurons, and have important implications for understanding the mechanisms of neurodegeneration due to perturbations in mitochondrial fusion.

400. Does mitochondrial fusion delay aging?

Hsiuchen Chen

Mitochondria possess a unique genome (mtDNA) that is critical for the function of the electron transport chain. The respiratory chain provides several functions, including the generation of ATP, maintenance of the mitochondrial membrane potential, and production of reactive oxygen species (ROS). One theory of aging hypothesizes that accumulation of mtDNA mutations leads to mitochondrial and thus cellular dysfunction. In support of this theory, mice carrying mutations in the proofreading subunit of mtDNA polymerase prematurely display a multitude of characteristics associated with aging, including reduced weight, hair loss, osteoporosis, anemia, and hearing loss. Most strikingly, these mice (termed POLG mice) also show reduced life spans.

Mitochondrial dynamics is crucial to preserving mitochondrial function. Loss of fusion leads to reduction in cellular respiration and membrane potential. In addition, disruption of fusion causes fragmentation of the mitochondria, resulting in a much greater number of mitochondria per cell, each with a significantly smaller mass. Anti-DNA stains show that whereas, nearly all wild-type mitochondria possess mtDNA nucleoids, the majority of fusion-deficient mitochondria lack mtDNA. Therefore, there is a sequestration of mtDNA into a much smaller mitochondrial mass. This mass may be insufficient to support adequate electron transport activity, thereby explaining the reduction of cellular respiration in the presence of mtDNA. It is important to note that with normal fusion, mitochondria can rapidly replenish stochastic loss of materials. However, in the absence of fusion, mitochondria lacking mtDNA have no pathway to regain either the mtDNA or the proteins they encode, and are therefore, permanently dysfunctional.

One interesting question is how the two pathways described above may intersect. If fusion does indeed protect against mtDNA loss, lack of fusion may exacerbate the aging phenotype seen in the POLG mice. To test this hypothesis, we are now mating the POLG mice to our

fusion-deficient mice. We will examine the time course of aging in the experimental mice, and use them to derive cell cultures in which to explore the cell biological consequences of accumulating mtDNA mutations in the absence of fusion.

401. Functional studies of Mfn2 alleles found in Charcot-Marie-Tooth disease

Scott Detmer

Mfn2, an oligomeric mitochondrial protein important for mitochondrial fusion, is mutated in Charcot-Marie-Tooth disease 2A (CMT2A), a peripheral neuropathy characterized by axonal degeneration. In addition to homo-oligomeric complexes, Mfn2 also associates with Mfn1, but the functional significance of such hetero-oligomeric complexes is unknown. Also unknown is why Mfn2 mutations in CMT2A lead to cell type-specific defects, given the widespread expression of Mfn2. Here, we show that homo-oligomeric complexes formed by many Mfn2 disease mutants are non-functional for mitochondrial fusion. Wild-type Mfn1, however, complements mutant Mfn2 through the formation of hetero-oligomeric complexes, including complexes that form in *trans* between mitochondria. Wild-type Mfn2 cannot complement the disease alleles. Our results highlight the functional importance of Mfn1/Mfn2 hetero-oligomeric complexes and the close interplay between the two mitofusins in control of mitochondrial fusion. Further, they suggest that tissues with low Mfn1 expression are vulnerable in CMT2A, and that methods to increase Mfn1 expression in the peripheral nervous system would benefit CMT2A patients.

402. Mouse models of Charcot-Maria-Tooth disease type 2A

Scott Detmer

We have established both transgenic and knock-in mouse models of CMT2A. We have generated multiple transgenic lines of mice expressing mutant Mfn2 under a motor neuron-specific promoter and have confirmed localized transgene expression. Interestingly, one of these lines exhibits a striking phenotype: affected mice are unable to dorsi-flex their hindpaws and subsequently display a gait defect characterized by dragging of the hindlimbs during walking. Over time this leads to deformities in the hindlimbs. This phenotype is remarkably similar to the symptoms of CMT2A patients. Such patients have distal limb weakness, which is manifested by an inability to dorsi-flex the foot, leading to "foot drop" during walking. Eventually this leads to foot deformities such as hammertoe caused by muscle imbalance.

In a second approach, we have generated knock-in mutations corresponding to two of the Mfn2 mutant alleles. In the heterozygous state neither allele manifests a behavioral phenotype. However, for one of the alleles, both homozygous mutant animals and animals with the mutant allele over a Mfn2 null allele develop an unsteady gait, are severely runted, and die by four weeks of age. We are characterizing both transgenic and knock-in mice for motor neuron function, fitness, neuronal histology, and neuronal mitochondrial morphology. We expect these to be valuable models for the study of CMT disease progression.

403. Structural and functional studies of the fission apparatus of mitochondria

Yan Zhang

Mitochondrial fission is an important process controlling mitochondrial physiology. First, mitochondrial fission regulates apoptosis in yeast, worms, flies, and mammals. Second, mitochondrial fission is an essential process, and reduced fission results in neonatal lethality with microcephaly in humans. Finally, defects in mitochondrial fission disrupt mitochondrial distribution in neurons and result in defective synaptic transmission.

Budding yeast is an excellent model to study mitochondrial fission. Mitochondrial fission in *Saccharomyces cerevisiae* is mediated by Dnm1p, Fis1p, Mdv1p, and Caf4p. During assembly of the mitochondrial fission complex, the mitochondrial outer membrane protein Fis1 recruits the dynamin-related protein Dnm1p to mitochondria via the molecular adaptors Mdv1p and Caf4p. Fis1p contains a tetratricopeptide repeat (TPR)-like domain, a well-known protein-protein interaction module. By analogy with other TPR domains, it has been presumed that Fis1p interacts with Mdv1p and Caf4p through a prominent hydrophobic groove on the concave surface of the TPR fold. We use biochemical and structural approaches to show that Fis1p employs a novel mechanism to recruit the adaptor proteins Mdv1p and Caf4p. In the Fis1-Caf4 crystal structure, Caf4p forms a helical clamp to wrap around both the convex and concave surfaces of Fis1. This binding mode is distinct from other TPR-containing proteins. Genetic and biochemical studies indicate that both binding interfaces are important for binding of adaptors to Fis1p, and for the ability of Fis1p to mediate mitochondrial fission *in vivo*. Our results provide structural insights into assembly of the mitochondrial fission complex and suggest that TPR domains can have diverse modes of ligand recruitment. Comparison of mammalian and yeast Fis1 structures suggests that mammalian Fis1 binds to ligands in a similar manner.

404. OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L

Zhiyin Zhang

OPA1, a dynamin-related GTPase mutated in dominant optic atrophy, is required for the fusion of mitochondria. Proteolytic cleavage by the mitochondrial processing peptidase generates long isoforms from eight mRNA splice forms, whereas, further cleavages at protease sites S1 and S2 generate short forms. Using OPA1-null cells, we developed a cellular system to study how individual *OPA1* splice forms function in mitochondrial fusion. Only mRNA splice forms that generate a long isoform in addition to one or more short isoforms support

substantial mitochondrial fusion activity. On their own, long and short OPA1 isoforms have little activity, but when co-expressed, they functionally complement each other. Loss of mitochondrial membrane potential destabilizes the long isoforms and enhances the cleavage of OPA1 at S1 but not S2. Cleavage at S2 is regulated by the i-AAA protease Yme1L. Our results suggest that mammalian cells have multiple pathways to control mitochondrial fusion through regulation of the spectrum of OPA1 isoforms.

405. Elucidation of the molecular mechanism of Mgm1/OPA1-induced membrane fusion in mitochondria

Tadato Ban

The dynamin-related GTPase OPA1/Mgm1 has been reported to play a central role in fusion of the inner membranes of mitochondria. However, little is understood about its biochemical mechanism. Other dynamin family proteins tubulate and constrict lipid membranes during endocytosis and vesicular trafficking. It is though that self-assembly into filamentous structures and activation of GTP hydrolysis is critical to these functions. It is unknown to what extent OPA1 shares these biochemical properties. Using purified, recombinant OPA1, we are developing an *in vitro* assay to study the effect of this protein on lipid membranes.

Publications

Chan, D.C. (2006a) Dissecting mitochondrial fusion. *Dev. Cell* **11**:592-594.

Chan, D.C. (2006b) Mitochondria: Dynamic organelles in disease, aging, and development. *Cell* **125**:1241-1252.

Chan, D.C. (2006c) Mitochondrial fusion and fission in mammals. *Ann. Rev. Cell & Dev. Biol*. **22**:79-99.

Chen, H. and Chan, D.C. (2006) Critical dependence of neurons on mitochondrial dynamics. *Curr. Op. Cell Biol*. **18**: 453-459.

Chen, H., McCaffery, J.M. and Chan, D.C. (2007) Mitochondrial fusion protects against neurodegeneration in the cerebellum. *Cell* **130**:548-562.

Choi, S.Y., Huang, P., Jenkins, G.M., Chan, D.C., Schiller, J. and Frohman, M.A. (2006) A common lipid links Mfn-mediated mitochondrial fusion and SNARE-regulated exocytosis. *Nature Cell Biol.* **8**:1255-1262.

Detmer, S.A. and Chan, D.C. (2007) Complementation between mouse Mfn1 and Mfn2 protects mitochondrial fusion defects caused by CMT2A disease mutations. *J. Cell Biol.* **176**:405-414.

Griffin, E.E. and Chan, D.C. (2006) Domain interactions within Fzo1 oligomers are essential for mitochondrial fusion. *J. Biological Chem*. **281**:16599-16606.

Griffin, E.E., Detmer, S.A. and Chan, D.C. (2006) Molecular mechanism of mitochondrial membrane fusion. *Biochimica et Biophysica Acta* **1763**:482-489.

Song, Z., Chen, H., Fiket, M., Alexander, C. and Chan, D.C. (2007) OPA1 processing controls mitochondrial fusion and is regulated by mRNA splicing, membrane potential, and Yme1L. *J. Cell Biol.* In press.

Zhang, Y. and Chan, D.C. (2007) New insights into mitochondrial fusion. *FEBS Letters* **581**:2168-2173.

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Summary: The Deshaies lab works on two basic biological processes: control of cell division, and regulation of cell function by attachment of ubiquitin or ubiquitin-like proteins to target polypeptides. We are particularly interested in how attachment of ubiquitin to proteins enables their degradation, and how protein degradation via this mechanism is used to regulate cell division.

Defective control of cell division can result in disease, as when unrestrained cell proliferation leads to cancer. Defects of the ubiquitin system can also lead to cancer, as well as neurodegenerative diseases. An understanding of how the cell division machinery and the ubiquitin system operate will thus, provide insight into basic cellular processes essential to the life of eukaryotic cells, and may suggest cures for diseases that affect millions of people.

We are using biochemical, molecular, and genetic approaches in baker's yeast and mammalian cells to investigate cell proliferation and the ubiquitin system. Our long-term goal is to understand how these processes work and how they are controlled. Baker's yeast is an excellent organism for basic cell biological studies because it is easy to work with, and many studies have confirmed that yeast and animal cells largely use the same proteins to regulate basic cellular processes.

The next section contains a brief description of the four major areas of investigation in the lab, followed by thumbnail descriptions of all current projects.

SCF ubiquitin ligases: Mechanism, regulation, and physiology

Cellular proteins are marked for degradation by attachment of the polypeptide ubiquitin. Ubiquitin is attached to substrates by a cascade comprising ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes. Ubiquitination occurs when an E3 enzyme binds to both substrate and E2 ubiquitin conjugating enzyme, bringing them into proximity so that ubiquitin is transferred from the E2 to substrate. Specificity and regulation of ubiquitination are typically imparted by E3s, which are the most diverse components of the system. Once ubiquitin is attached to a substrate, the reaction can either terminate (in which case the ubiquitin serves as a regulatory signal to modulate protein function or localization) or continue, leading to the assembly of a multiubiquitin chain. A chain of four ubiquitins suffices to specify destruction of the substrate by the proteasome.

Mechanism of action of SCF ligases: In 1999, we and others reported that RING domains underlie ubiquitin ligase activity (Seol *et al*., 1999). This discovery revealed what is now thought to be the largest class of ubiquitin ligases, with up to 385 members. The progenitor of the RING-based ubiquitin ligases, SCF (Feldman *et al*., 1997), defines a subfamily of multisubunit cullin–RING ligases that may number as many as 300-350 members, due to combinatorial mixing of subunits. Thus, there may be as many as 700 distinct RING ligase complexes, which would make it the largest-known family of enzymes in human cells (Petroski and Deshaies, 2005). As befits such a large family, the cullin–RING ligases have been implicated in a dazzling array of cellular and organismal processes, ranging from circadian rhythms to sulfur assimilation. However, despite the biological import of these enzymes, the mechanism of how they work remains unknown.

Over the past few years, we have made substantial progress towards understanding how SCF enzymes work. A key step was to develop a reconstituted system in which a physiological substrate (budding yeast Cdk inhibitor Sic1 assembled into cyclin-Cdk complexes) is ubiquitinated by a complex of SCF and the ubiquitin-conjugating enzyme Cdc34, and subsequently is degraded by the proteasome – work that was carried out by Renny Feldman, Craig Correll, and Rati Verma (Feldman *et al*., 1997; Verma *et al*., 2001). Matt Petroski then constructed Sic1 substrates bearing single ubiquitin acceptor lysines, and used these substrates to characterize the impact of ubiquitin chain position on substrate recognition and degradation by the proteasome (Petroski and Deshaies, 2003). Matt went on to use his single-lysine substrate to show that assembly of a ubiquitin chain can be broken down into distinguishable initiation and elongation reactions (Petroski and Deshaies, 2005).

We believe that the system that we have developed is the best available for studying biochemical mechanisms of ubiquitination, because our substrate is multiubiquitinated and degraded at rates that approach those that must occur *in vivo*, and the nature of the reaction products is defined due to the substrate having only a single lysine acceptor. We are now in an excellent position to exploit our *in vitro* systems to address basic questions that are of central importance to understanding the mechanisms that underlie the operation of the ubiquitin-proteasome system (UPS) (see abstracts by G. Kleiger, N. Pierce, and A. Saha). For example, how does a RING domain activate ubiquitin transfer from ubiquitin-conjugating enzyme to substrate? What is the basis for the synthesis of the lysine 48-linked ubiquitin chains that

signal proteolysis? How is processive ubiquitination of Sic1 achieved, and how does it relate to the dynamics of substrate and Cdc34 (E2) association with SCF? How does ubiquitin transfer occur across an ~50 Å gap that is thought to exist between the substrate and E2 enzyme bound to SCF? How do unique sequence elements of Cdc34 – including its tail and the acidic loop insertion sequence near the catalytic site – contribute to its function with SCF? None of these questions are resolved for any RING E3, and thus, illuminating the answers will establish paradigms that inform our understanding of how hundreds of ubiquitin ligase enzymes work. The insights that emerge from this effort may also provide clues to the development of drugs that modulate the activity of RING-based ligases.

Regulation of SCF ubiquitin ligases: It was originally thought that SCF ubiquitin ligases are constitutively active, and substrate turnover is regulated by phosphorylation of the substrate. Subsequently, it was shown that the Cul1 subunit of SCF is modified covalently by the ubiquitin-like protein Nedd8, thus raising the possibility that SCF might be regulated post-translationally. In 2001, two students from the lab, Svetlana Lyapina and Greg Cope, reported that a poorly understood protein complex known as COP9 Signalosome (CSN) binds SCF in animal cells, and promotes the cleavage of Nedd8 from Cul1 via a tightly-associated Nedd8 isopeptidase activity (Lyapina *et al.*, 2001). This was the first biochemical function ascribed to CSN, and opened the door to the study of SCF regulation by reversible cycles of 'neddylation.' Subsequently, Greg Cope discovered that the Csn5 subunit harbors a motif that we named 'JAMM' (for JAb1/Mpn domain Metalloenzyme) (Cope *et al*., 2002). We predicted that JAMM comprises a novel metalloprotease active site. Later, Xavier Ambroggio, who was a joint student with Doug Rees in Chemistry, substantiated this prediction by employing X-ray crystallography to show that the conserved residues of the JAMM motif coordinate a zinc ion in an active site-like cleft of the protein AF2198 from *Archaeglobulus fulgidis* (Ambroggio *et al.*, 2004). We continue to investigate the regulation of SCF by CSN (see abstract by E. Emberley). We hope to understand how CSN itself is controlled, and what role CSN plays in sustaining active SCF complexes and sculpting the repertoire of SCF complexes in a cell.

Mechanism of action and regulation of the 26S proteasome

Once substrates are ubiquitinated by E3s, they are degraded by the 26S proteasome. The 26S proteasome is a large protein machine that comprises two major subcomplexes: the 20S 'core' proteasome and the 19S regulatory 'cap.' The 20S core forms a cylindrical structure that houses the protease active sites of the proteasome. Each end of the 20S cylinder is decorated with a 19S cap. The 19S cap can be further subdivided into the 'lid' and the 'base.' The base contains six ATPases that are thought to form a ring that abuts the end of the 20S cylinder. The lid, in turn, sits upon the base. The base is thought to control access of substrates into the 20S proteolytic chamber, whereas, the lid confers ubiquitin dependence. The 26S proteasome degrades proteins that are linked to a chain of at least four ubiquitins. The tetraubiquitin chain mediates binding of the attached substrate to the proteasome, after which it is disengaged from bound partners, unfolded, deubiquitinated, and translocated into the proteolytic chamber of the proteasome where the denuded substrate is degraded.

A fully reconstituted system to study Sic1 degradation: To harness the power of yeast molecular genetics to enable dissection of the mechanism-of-action of the proteasome, we developed a system wherein ubiquitinated Sic1 generated *in vitro* with recombinant $SCFC^{dc4}$ is degraded by affinity-purified yeast proteasomes (Verma *et al.*, 2001). Remarkably, purified proteasomes can extract ubiquitinated Sic1 from complexes with S phase cyclin-Cdk, degrade the Sic1 and release active S phase cyclin-Cdk protein kinase. This result emphasized that the proteasome has the intrinsic ability to disassemble protein complexes to selectively degrade ubiquitinated substrates, and set the stage for our subsequent studies on substrate targeting and deubiquitination.

Role of deubiquitination in the degradation of Sic1: In the course of characterizing the degradation of ubiquitinated Sic1, we noticed that when the 20S protease inhibitor epoxomicin was present, ubiquitinated Sic1 was converted to a completely deubiquitinated species (Verma *et al.*, 2002). Fortuitously, at the same time as this we observed that the CSN – which is related to the lid subcomplex of the proteasome 19S cap – cleaves the ubiquitin-like protein Nedd8 from the Cul1 subunit of SCF. Spurred by this confluence of observations, we demonstrated that the Rpn11 subunit of the proteasome lid contains a putative JAMM metalloprotease active site analogous to that of Csn5, and this motif is essential for the deubiquitination of Sic1 *in vitro* and the degradation of multiple UPS substrates *in vivo*.

Multiubiquitin chain receptors target substrate to the proteasome: Although it has long been clear that a multiubiquitin chain targets an appended substrate to the proteasome for degradation, the mechanism of targeting has remained poorly understood. Genetic studies in yeast have suggested a potential role for multiubiquitin chain-binding proteins, including Rad23, Dsk2, Ddi1, and Rpn10. In contrast, biochemical studies in mammalian systems have emphasized a role for the proteasome ATPase Rpt5 as a multiubiquitin chain receptor, and have suggested that proteins such as Rad23 prevent premature metabolism of substrate-linked ubiquitin chains. We reasoned that our reconstituted system would enable us to address this fundamental question from a functional, mechanistic perspective. We first demonstrated using mutant proteasomes and add-back experiments that Rad23 and Rpn10 play a direct role in targeting ubiquitinated Sic1

to the proteasome for degradation (Verma *et al*., 2004a). We then went on to show that the multiubiquitin chain receptor activities of Rad23 and Rpn10 play a redundant role in sustaining turnover of Sic1 *in vivo*. Surprisingly, individual deletion of these and other receptor proteins led to the accumulation of different UPS substrates, suggesting that the receptors define a layer of specificity that resides downstream of the E3s and upstream of the proteasome. This hypothesis opens up a host of interesting questions about how specificity is achieved in the targeting step, and what its biological purpose is. We plan to address these key questions over the next several years using a combination of biochemical, molecular genetic, and proteomic approaches (see abstract by T. Gomez).

Proteasome inhibitors: Small molecules that inhibit protein turnover by the proteasome can selectively kill cancer cells. One inhibitor has already been approved by the FDA for the treatment of the blood cell cancer multiple myeloma, and others are currently in clinical development. We are interested to understand why these inhibitors kill some cancer cells (such as multiple myeloma and other hematological cancers) but are much less effective against solid tumors (see abstract by S. Radhakrishnan).

A couple of years ago, in collaboration with Dr. Randy King at Harvard, we discovered ubistatins, which are novel inhibitors of protein degradation by the proteasome. We demonstrated that ubistatin A binds to the ubiquitin chain in the same intersubunit cleft that is normally bound by the multiubiquitin chain receptors (Verma *et al*., 2004b). This binding prevents the ubiquitin chain from binding to receptors (Rpn10 and Rad23) that link it to the proteasome. A postdoctoral fellow who is just joining the lab (E. Miller) plans to study a second ubistatin (ubistatin B) that emerged from this screen. Ubistatin B shows promising activity in blocking protein degradation by the UPS in mammalian cells. We believe that ubistatins will be useful tools for studies on the UPS.

Role of Cdc48 in targeting and degradation of ubiquitinated proteins: Recently, we have become intrigued by a poorly understood protein, Cdc48, that, like the ubiquitin chain receptors, operates downstream of ubiquitin ligases to promote degradation of ubiquitinated proteins by the proteasome. The role of Cdc48 in protein turnover was originally thought to be confined to pulling malfolded secretory proteins through the endoplasmic reticulum membrane so that they can be degraded by the proteasome. However, several lines of evidence hint at a far broader role. Interestingly, there may be as many as seven distinct Cdc48 complexes in budding yeast, and our unpublished data points to the existence of ~15 distinct Cdc48 complexes in human cells (see abstract by G. Alexandru). Why all of this complexity? It is difficult to even begin to answer this question, because we know so little about Cdc48's function apart from its role in translocation across the ER membrane. Whatever Cdc48 is doing, it appears to be a fundamental component of the UPS, and thus, understanding how it works is important. We plan to attack this problem by first identifying substrates whose degradation depends on particular Cdc48 complexes. One strategy is to study candidate substrates such as Hsl1 or Cdc5 (see abstract by R. Verma). Another strategy is to employ the mass spec-based proteomics technology discussed below, as well as a novel *in vivo* substrate screen (see abstract by T. Chou). We will then reconstitute the degradation of these substrates using defined components. The objective will be to develop a reconstituted system in which turnover of the substrate is dependent upon Cdc48. We will then use this system to establish the mechanism-of-action of Cdc48. Armed with this information, we will be in a position to initiate investigations on how ubiquitin receptors and Cdc48 complexes collaborate to enable degradation of ubiquitinated substrates. Given the diversity of receptors that guide ubiquitinated proteins to the proteasome and the diversity of Cdc48 complexes that appear to act in concert with these receptors, there is clearly much about the targeting and degradation of ubiquitinated proteins that we do not understand, and thus, this topic may be fertile ground for making unexpected discoveries.

Proteomics

The Yates laboratory at Scripps has developed a method dubbed "multidimensional protein identification technology" (MudPIT). In this method, a complex mixture of proteins is digested with protease and the resulting peptides are fractionated by two-dimensional chromatography on a column that is in-line with the electrospray interface of a mass spectrometer. Johannes Graumann and Thibault Mayor have applied MudPIT to the study of ubiquitination in yeast. In our first efforts we employed subtractive comparisons of samples from wild-type and mutant cells to identify the ubiquitinated polypeptides that accumulate when the Rpn10 multiubiquitin chain receptor is absent (Mayor *et al*., 2005). More recently, we have used differential labeling of mutant and wild-type cells with stable isotopes to obtain quantitative estimates of substrate accumulation in Rpn10-deficient cells (Mayor *et al*., 2007). By identifying the set of substrates whose abundance is altered when a particular ubiquitin pathway component is mutated or blocked by the action of a drug, we hope to gain insight into enzyme-substrate relationships, which in turn may yield insights into the mechanisms that underlie specificity. Moreover, knowledge of the substrates affected can provide clues to the phenotypes that may occur upon inactivation of a particular component. Finally, the ability to quantify substrate accumulation may enable us to see subtle defects, such as those that occur when one member of a redundant pair of enzymes is mutated.

In a second project that we are just starting, we plan to use a combination of crosslinking and MudPIT to identify dynamic protein interactions that occur inside cells but do not survive the immunoprecipitation and washing steps that are normally employed in the affinity

purification of protein complexes for analysis by mass spectrometry (see abstract by E. Lee).

A third proteomics-related project is to screen an shRNA library to identify genes of the UPS that influence the differentiation of embryonic stem cells into cardiomyocytes (see abstract by N. Honarpour).

Functions of the RENT complex in cell cycle control and nucleolar biogenesis

Several years ago, a graduate student, Wenying Shou, discovered the RENT complex, and proposed that the mitotic exit network (MEN) specifies the exit from mitosis in budding yeast by promoting disassembly of RENT (Shou *et al.*, 1999). RENT is comprised of the nucleolar anchor protein Net1, the cell cycle regulatory protein phosphatase Cdc14 and the chromatin silencing protein Sir2. Cdc14 is required for the exit from mitosis, which it promotes by dephosphorylating (and thereby activating) proteins that mediate the inactivation of cyclin/CDK activity at the end of mitosis. Throughout the cell cycle, Cdc14 is confined to the nucleolus through its interaction with Net1. At the end of mitosis, the successful completion of anaphase activates the MEN signaling pathway, which disengages Cdc14 from Net1. The emancipated Cdc14 goes on to inactivate cyclin/CDK and thereby trigger the exit from mitosis. This hypothesis for how the exit from mitosis is controlled in budding yeast was dubbed 'RENT control' by Shou, *et al*., 1999. Over the past few years, it has become apparent that RENT is disassembled by a two-step mechanism. In early anaphase, Cdc14 is released from Net1 through a novel activity of separase. Separase is a protease that activates chromosome segregation in anaphase by cleaving the cohesin protein that holds sister chromatids together.

Throughout interphase and early mitosis, separase activity is repressed by a tightly bound inhibitor, securin. At the metaphase-anaphase boundary, securin is abruptly degraded, thereby liberating separase to cleave cohesin and initiate chromosome segregation. In addition to being a protease, separase has a second activity that promotes the phosphorylation of Net1 by cyclin B–Cdk. A graduate student in the lab, Ramzi Azzam, had identified this phosphorylation and demonstrated that it induces the dissociation of Cdc14 from Net1 (Azzam *et al.*, 2004). Thus, the action of separase links initiation of the exit from mitosis with the initiation of chromosome segregation. In late anaphase the MEN serves to sustain Cdc14 release and enable the released Cdc14 to gain access to the cytoplasm, such that its substrates are dephosphorylated and the cell exits mitosis. Our current goal is to understand how the terminal signaling component of the MEN, the protein kinase Dbf2, mediates the sustained release of Cdc14 from the nucleolus and its translocation to the cytoplasm thereby triggering exit from mitosis (see abstract by D. Mohl).

The following Deshaies Lab publications were cited in the preceding overview:

Ambroggio, X.I., Rees, D.C. and Deshaies, R.J. (2004) JAMM: A metalloprotease-like zinc site in the proteasome and signalosome. *PLoS Biol.* **2**(1):E2.

Azzam, R., Chen, S.L., Shou, W., Mah, A.S., Alexandru, G., Nasmyth, K., Annan, R.S., Carr, S.A. and Deshaies, R.J. (2004) Phosphorylation by cyclin B-Cdk underlies release of mitotic exit activator Cdc14 from the nucleolus. *Science* **305**(5683):516-519.

Cope, G.A., Suh, G.S., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V. and Deshaies, R.J. (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science* **298**(5593):608-611.

Feldman, R., Correll, C., Kaplan, K. and Deshaies, R.J. (1997) A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* **91**:221-230.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N. and Deshaies, R.J. (2001) Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* **292**(5520):1382-1385.

Mayor, T., Lipford, J.R., Graumann, J., Smith, G.T. and Deshaies, R.J. (2005) Analysis of poly-ubiquitin conjugates reveals that the Rpn10 substrate receptor contributes to the turnover of multiple proteasome targets. *Mol. Cell Proteomics* **4**:741-751.

Petroski, M.D. and Deshaies, R.J. (2003) Context of multiubiquitin chain attachment influences the rate of Sic1 degradation. *Mol. Cell.* **11**(6):1435-1444.

Petroski, M. and Deshaies, R.J. (2005) Mechanism and regulation of cullin-RING ubiquitin ligases. *Nature Rev. Mol. Cell Biol.* **6**:9-20.

Petroski, M.D. and Deshaies, R.J. (2005) Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell* **123**:1107-1120.

Seol, J., Feldman, R., Zachariae, W., Shevchenko, A., Correll, C., Lyapina, S., Chi, Y., Galova, M., Claypool, J., Sandmeyer, S., Shevchenko, A., Nasmyth, K. and Deshaies, R.J. (1999) Cdc53/cullin and the essential Hrt1 RING-H2 subunit of SCF define a ubiquitin ligase module that activates the E2 enzyme Cdc34. *Genes Dev.* **13**:1614-1626.

Shou, W., Seol, J.-H., Shevchenko, A., Baskerville, C., Moazed, D., Shevchenko, A., Charbonneau, H. and Deshaies, R.J. (1999) The exit from mitosis is triggered by Tem1-dependent release of Cdc14 protein phosphatase from nucleolar RENT complex. *Cell* **97**:233-244.

Verma, R., McDonald, H., Yates, J.R. III, and Deshaies, R.J. (2001) Selective degradation of ubiquitinated Sic1 by purified 26S proteasome yields active S phase cyclin-Cdk. *Mol. Cell* **8**(2):439-448.

Verma, R., Aravind, L., Oania, R., McDonald, W.H., Yates, J.R. III, Koonin, E.V. and Deshaies, R.J. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* **298**(5593):611-615.

Verma, R., Oania, R., Graumann, J. and Deshaies, R.J. (2004a) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell* **118**(1):99-110.

Verma, R., Peters, N., D'Onofrio, M., Tochtrop, G., Sakamoto, K., Varadan, R., Zhang, M., Coffino, P., Fushman, D., Deshaies, R.J. and King, R. (2004b) Ubistatins inhibit proteasome-dependent degradation by binding the ubiquitin chain. *Science* **306**:117-120.

406. p97 regulation via interaction with a variety of p47-related co-factors

Gabriela M. Alexandru

The 97 kDa valosin-containing protein (p97 or VCP) is a type II AAA (ATPase associated with a variety of activities) ATPase, highly conserved from archaebacteria to mammals. p97 plays a role in seemingly unrelated cellular activities, such as membrane fusion, endoplasmic reticulum-associated degradation (ERAD) and cell cycle regulation. All of these functions involve recognition of ubiquitinated protein-substrates and, at least in some cases, their subsequent degradation by the proteasome. In its active form, p97 forms homohexameric barrel structures in which the N-termini are free to bind specific co-factors. Thus, p97 in complex with p47 is thought to regulate membrane fusion, while p97/Npl4/Ufd1 complexes are mainly required for ERAD. In an attempt to further understand the molecular basis of p97's diverse functions we have analyzed p97 immunoprecipitates from human tissue culture cells by MuDPIT mass spectrometry, searching for new p97 co-factors. This analysis revealed a whole group of previously unidentified p97 binding partners, all sharing a domain structure similar at least in part to p47. Two of them have been linked to human diseases, such as atopic dermatitis and alveolar soft part sarcoma. However, the biological function for most of these proteins is largely unknown. Comparative analysis of immunoprecipitates from human cells transfected with a Flag-tagged version of each of the newly identified co-factors revealed that some of them can bind ubiquitinated proteins and also interact with the ERAD p97 co-factors, Npl4 and Ufd1, suggesting they might also be involved in ubiquitin-dependent protein degradation. This analysis also revealed potential substrates interacting specifically with particular p97 co-factors. Their true nature as p97 substrates will be confirmed by studying their stability in cells siRNA-depleted for p97 or the corresponding p97 co-factor. The confirmed substrates will subsequently be used to gain mechanistic insight into the role of p97 co-factors in regulating p97 function.

407. Development of thiol-reactive inhibitors for Cdc48/P97 AAA ATPase

Tsui-Fen Chou

Cdc48/p97 is an important AAA ATPase not only due to its intriguing diverse cellular functions but also because it has been implicated in mediating turnover of many proteins involved in tumorgenesis. In an effort to develop small-molecule inhibitors for Cdc48/p97 based on its X-ray structure, we first searched for the scaffolds that are likely to bind to the ATP binding pocket of the D2 domain of murine p97. Of particular interest to us are scaffolds that include an electrophile that can readily react with a natural active-site cysteine of p97 or with a cysteine engineered into the active site of yeast Cdc48 (CDC48-T532C). Inhibition is monitored by using an *in vitro* ATPase activity assay and the exact cysteine residue involved in the inactivation will be confirmed by mass spectroscopy. *In vivo* inhibition potency will be compared in a yeast strain (*cdc48Δ*) containing plasmids that express wild-type Cdc48 or T532C-Cdc48 to evaluate the selectivity of the thiol-reactive compounds. Compounds identified from this study that show good inhibition potency toward the wild-type enzyme will be useful lead compounds for developing anticancer drugs that target the ubiquitin-proteasome system at a stage upstream of the proteasome. On the other hand, those compounds that inhibit engineered mutants but not the wild-type yeast enzyme will provide a bio-orthogonal inhibitor/mutant pair that can serve as a valuable biological tool for study the physiological function and mechanism of Cdc48/p97.

408. Regulation of SCF ubiquitin ligase activity

Ethan Emberley

Ubiquitin-mediated protein degradation has emerged as a pivotal process in many areas of cell biology. The multi-subunit SCF (Skp1/Cul1/F-box protein) ubiquitin ligase enzymes transfer ubiquitin molecules onto target proteins destined for degradation by the 26S proteosome. Many cellular proteins, possibly hundreds, are targeted for degradation by SCF complexes. Inappropriate SCF activity can result in an enhancement in the degradation of a pool of proteins that may be required to maintain the cell in a normal state. As well, the SCF may no longer ubiquitinate a separate pool of substrates resulting in their intracellular accumulation and amplification of their function. Despite impressive advancements in our knowledge about the SCF protein complex, many fundamental aspects remain poorly understood, including its mechanism of action and regulation. We have begun to study the mechanism by which substrate binding to its respective F-box protein controls SCF activity. We propose that substrate binding either positively regulates the attachment of the ubiquitin-like protein Nedd8 to the SCF subunit Cul1 by Ubc12 (i.e., neddylation), or negatively regulates Cul1 deneddylation by the COP9 signalosome. The Nedd8 modification on Cul1 is necessary for SCF ubiquitin ligase activity and provides another layer of SCF regulation. The SCF and COP9 signalosome complexes have been previously

shown to physically interact with each other and we aim to define the importance of this interaction further by characterizing the biochemical requirements that result in the removal of Nedd8 from Cul1 and the eventual shutdown of SCF's ubiquitin ligase activity. By describing the specific effects of substrate binding on the neddylation status of SCF, we will better understand the steps needed for SCF activity, as well as described a mechanism by which the protein to be degraded is influencing its own ubiquitination. This new pathway controlling SCF activity could be the target of therapeutic intervention as deregulated SCF activity has been suggested to be involved in several human malignancies.

409. Binding of 26S proteasome subunits to ubiquitin receptor proteins

Tara Gomez

The mechanism by which ubiquitinated proteins are delivered to the 26S proteasome is poorly understood. The 26S proteasome is composed of a 20S catalytic core particle (CP) and a 19S regulatory particle (RP), which itself is composed of a base and a lid. In *Saccharomyces cerevisiae*, the base is composed of about eight proteins, one of which, *RPN1* is known to play an important role in binding to ubiquitin receptor proteins such as *RAD23*, *DSK2* and *UBP6*, all of which contain a ubiquitin-like domain (UBL) (1, 2). It is believed that *RAD23* and *DSK2* deliver ubiquitinated substrates to the proteasome to promote degradation, and may bind the same or overlapping sites of *RPN1* via their UBL domain (3,4,5). *UBP6*, a deubiquitinating enzyme, may preferentially deubiquinate substrates targeted by *RAD23*, but does not compete with *RAD23* for binding to *RPN1* (3). We plan to take a genetic approach to gain a better understanding of the mechanism and regulation of how UBL domain proteins are recruited to the proteasome.

References

1. Seeger *et al.* (2003) *JBC* **278**(19):16791-16796.
2. Elsasser *et al.* (2002) *Nature Cell Biol.* **4:**725-730.
3. Elsasser *et al.* (2004) *Nature Cell Biol.* **7:**742-749.
4. Verma *et al.* (2004) *Cell* **118**(1):99-110.
5. Elsasser *et al.* (2004) *JBC* **279 (**26):26817-26822.

410. Ubiquitination in stem cell differentiation and cardiovascular development

Narimon Honarpour

Stem cell therapy is a developing technology with great potential to treat human disease. A major limitation, however, is that little is known about how stem cells differentiate. Thus, it is not currently possible to reliably generate tissue that could be used for cell-based therapy. Because the ubiquitin-proteosome system (UPS) plays a central role in regulating intracellular signaling, we hypothesize that key switches governing differentiation pathways are also controlled by the UPS. We propose to test this hypothesis by seeking UPS genes that influence embryonic stem cell differentiation into cardiovascular tissue. Our approach involves generating mouse embryonic stem cell lines that express lineage-specific reporter genes, transducing these cells with an shRNA library, and screening for spontaneous or accelerated differentiation. After identifying shRNAs that promote differentiation, mechanisms by which silenced UPS genes affect differentiation will be further analyzed. Current efforts are directed towards generating and characterizing a reporter cell line that can be used to screen for cardiac lineage commitment.

411. Molecular mechanism of ubiquitin chain synthesis by Cdc34

Gary Kleiger

Cdc34 is a ubiquitin conjugating enzyme that is an essential component of the Ubiquitin Proteasome System (UPS). It functions by catalyzing the formation of polyubiquitin chains onto protein substrates destined for degradation by the 26S proteasome. Cdc34 works in concert with the ubiquitin ligase SCF, which is also an essential component of the UPS.

Cdc34 and SCF combine to form a potent ubiquitin ligase complex that processively labels its substrates. This processivity has important functional consequences for living cells, since it has been shown that a protein substrate must be labeled with at least four consecutive ubiquitins before it is recognized by the proteasome. In addition, living cells contain proteins that degrade ubiquitin chains. Therefore, ubiquitin ligases are working against the clock to put enough ubiquitins onto their substrates so that they will be degraded.

Our goal is to explain the molecular mechanism of Cdc34 and SCF processive enzyme activity. We are focused on the C-terminal tail domain of Cdc34, a conserved acidic stretch of 80 to 120 amino acids, found on all Cdc34 molecules from yeast to humans. We, and others, have demonstrated that deletion of the Cdc34 tail either drastically reduces or eliminates all SCF-dependent Cdc34 activity. We have expressed and purified the tail domain by itself in bacteria cells. The addition of free tail to SCF-dependent Cdc34 reactions competes with wild-type Cdc34, demonstrating that the function of the tail is at least partially retained in the absence of the catalytic domain of Cdc34. This result also hints that the function of the tail is to interact with SCF.

If the function of the Cdc34 tail domain is to mediate an interaction between Cdc34 with SCF, we hypothesized that the molecular fusion of the Cdc34 catalytic domain without its tail sequence to the SCF ligase should rescue the tail delete phenotype. Currently our data support this notion and future directions are focused on both the further biochemical characterization of the tail, as well as characterization of the SCF-Cdc34 fusion constructs.

412. Identifying molecular components involved in Parkinson's disease

J. Eugene Lee

Parkinson's disease is the most prevalent neurodegenerative movement disorder. Familial-linked genes have provided novel insights into its pathogenetic mechanism. One of these genes is *parkin*, which encodes a 465-residue ubiquitin ligase. With the aid of ubiquitin-conjugating proteins, ubiquitin ligases catalyze the synthesis of a polyubiquitin chain on the target substrate, earmarking it for degradation. Currently, it is unclear which proteins are targeted by *parkin* for modification. Moreover, the enzymatic function of *parkin* is controversial. Certain lines of evidence suggest that *parkin* mediates K63-linked polyubiquitination, which signals cellular events that are distinct from those associated with the typical degradation-destined K48-linked polyubiquitination. Elucidation of the mechanism underlying Parkinson's disease will greatly benefit from a better understanding of how *parkin* functions. To address this issue, we will utilize a combination of mass spectrometry-based proteomics and *in vivo* cross-linking techniques. The specific goals are to identify the substrates and the ubiquitin-conjugating enzyme that are recruited by *parkin in vivo*. The result of these studies will shed light on how an aberration in the ubiquitin-proteasome system leads to the onset of a neurodegenerative disease.

413. Regulation of *Saccharomyces cerevisiae* Cdc14

Dane Mohl

Our work hopes to illuminate the mechanisms that allow the cell to sense the duplication of the DNA genome, and link completion of chromosome segregation to the initiation of cell division. In my research, I have used the model yeast system *Saccharomyces cerevisiae* to look more closely at the regulation of a key cell cycle phosphatase, Cdc14. Our work has demonstrated that a protein kinase complex called Dbf2/Mob1, that becomes active when each of the two new nuclei are segregated to opposite compartments of a pre-divisional cell, directly regulates Cdc14 phosphatase, thereby linking genome duplication to cytokinesis.

The aim of my project has been the identification of *cis*-acting sequences within Cdc14 and its inhibitor Net1p that are required for late mitotic regulation. We have also been searching for the transacting factors that act upon those epitopes. Live cell imaging and GFP fluorescence localization led us to conclude that a small portion of Cdc14-GFP visited the daughter bound spindle pole bodies of anaphase cells and could therefore, be a direct substrate of Dbf2/Mob1. Our research has, therefore, focused on identifying sequences within Cdc14 that could be sites of Dbf2/Mob1 phosphorylation. Mass spec peptide analysis of *in vitro*- and *in vivo*-phosphorylated Cdc14p has illuminated the importance of key C-terminal sequences within Cdc14. Genetic analysis and GFP fluorescence studies have strengthened our conclusion that Cdc14 is a direct substrate of Dbf2/Mob1 and that phosphorylation of Cdc14 within a tightly regulated nuclear localization signal may enable Cdc14 to escape nucleolar sequestration by Net1 thereby allowing it to reach targets within the cytoplasm.

Our most recent work hopes to find additional Dbf2/Mob1 substrates, as well as identify factors that may act as Cdc14 substrates or factors that partner with Cdc14 to facilitate its activity in both the cytoplasm and nucleus.

414. Integrated dynamics of Sic1

Nathan Pierce

Cell cycle progression in yeast is controlled by the activation and degradation of cyclins and cyclin-dependent kinases (Cdk). Our work focuses on the integrated dynamics of the B-type cyclin inhibitor Sic1. Sic1 is expressed at the end of mitosis, whereupon it binds and inhibits the protein kinase activity of Clb-Cdc28 Cdk complexes. Sic1 is multi-phosphorylated at the end of G1 phase by G1 cyclin (Cln)-Cdc28 Cdk complexes and is subsequently ubiquitinated by the SCFCdc4 ubiquitin ligase.

Multi-ubiquitination of Sic1 targets it to the 26S proteasome for degradation. Declining levels of Sic1 unmask the kinase activity by Clb-Cdc28, thereby driving cells to enter S phase and replicate their DNA. The sequential phosphorylation, ubiquitination, and degradation of Sic1 define the G1/S phase transition. Our goal is to elucidate the kinetics of each event individually and together *in vitro* to better understand how they might occur *in vivo*.

415. Proteasome inhibition as an anti-cancer strategy

Senthil K. Radhakrishnan

Recent studies have indicated that proteasome inhibition could be an attractive strategy in anti-cancer therapy. However, one potential problem with inhibiting the proteasome is the existence of a feedback loop that results in proteasome resynthesis and recovery. We hypothesize that blocking this feedback loop by downregulating proteasome resynthesis could increase the efficacy of proteasome inhibitor therapy. We are currently investigating the covalent, irreversible proteasome inhibitor YU101 in combination with a transcriptional or a translational inhibitor (to block proteasome resynthesis) in cancer cell culture models. If promising, this approach could be extended to a mouse model harboring human xenograft tumors. As a part of this project we also intend to test if pulse treatment with YU101 (which mimics the clinical situation where proteasome inhibitors are rapidly cleared from the plasma) followed by inhibition of proteasome resynthesis can effectively sustain proteasome inhibition as a whole. Overall, our approach could lead to rational drug combinations and hence effective therapies against cancer.

416. Targeting cancer-promoting proteins for ubiquitination and degradation

Agustin Rodriguez

We are developing a new approach to cancer therapy that exploits the ubiquitin-dependent proteolytic system of eukaryotic cells. This approach employs cell-permeable molecules that bind to the substrate-docking site of an ubiquitin ligase. Covalently linking such a molecule to compounds that bind to a desired target protein yields a heterobifunctional compound that we refer to as a ProTac (Proteolysis Targeting Chimeric Pharmaceuticals). ProTacs can be used, in theory, to trigger the destruction of any protein for which there exists a small, cell-permeable ligand. As "proof of principle," we designed a ProTac that contains a peptide that binds with high affinity to the substrate-docking domain of the ubiquitin ligase $SCF^{\delta\text{-}TRCP}$. We then chemically linked this peptide to the small molecule ovalicin, which binds covalently and specifically to the cellular enzyme methionine aminopeptidase-2 (MetAP-2). We demonstrated that the resulting peptide-ovalicin (ProTac) tethers MetAP-2 to $SCF^{\delta\text{-}TRCP}$, and targets MetAP-2 for ubiquitination and degradation[1]. To determine whether ProTacs could recruit different substrates to the $SCF^{\delta\text{-}TRCP}$ ubiquitin ligase for ubiquitination through non-covalent interactions, we generated ProTacs containing the same ligase-binding peptide linked to either estradiol or testosterone. Both the estrogen receptor (ER) and androgen receptor (AR) have been shown to enhance growth of breast and prostate cancer, respectively. We demonstrated that ProTacs containing estradiol or dihydroxytestosterone can trigger the degradation of ER and AR, respectively[2]. Making a cell-permeable ProTac was the next step to induce degradation of a target in living cells. The latest-generation ProTaCs are based on a hydroxyproline-containing peptide from HIF-1 that binds to the VHL ubiquitin ligase complex. These new ProTaCs based on the HIF-1 peptide induced the degradation of AR and ER in tissue culture cells[3]. Induced degradation of ER arrested cell proliferation of estrogen-dependent breast cancer cells as a consequence of down regulation of ER signaling. On the other hand, breast cancer cell lines that do not express ER and are not dependent upon ER signaling for proliferation were not affected by treatment with ProTaCs. We are now focusing on developing ProTacs into effective drugs for cancer treatment *in vivo*.

References

1. Sakamoto *et al.* (2001) *Proc. Natl. Acad. Sci. USA* **98**:8554-8559.
2. Sakamoto *et al*. (2003) *Mol. Cell Proteomics* **2**:1350-1358.
3. Schneekloth *et al.* (2004) *J. Am. Chem. Soc.* **126**:3748-3754.

417. Mechanism of ubiquitin chain assembly by SCF ligases

Anjanabha Saha

Protein modification by ubiquitination is mediated by ubiquitin ligases (E3) that include the highly diverse and extensive superfamily of cullin-RING ligases (CRLs). CRLs play a central role in various cellular and organismal processes. Understanding the function of E3 ligases is important, since E3 specificity determines which substrate is to be ubiquitinated and hence, it regulates the activity of specific proteins. We are utilizing the human SCF complex to investigate how the dynamics of substrate and E2 (ubiquitin conjugating enzyme) association with E3 regulate the specificity and the processivity of ubiquitination. These studies will aid in establishing how SCF distinguishes substrates from non-substrates and provide insight into how SCF ligases achieve specificity *in vivo*. In addition, we are also examining how covalent modification of CRLs by Nedd8 affects the kinetics of substrate ubiquitination.

418. Receptor pathways of the Ubiquitin Proteasome System (UPS)

Rati Verma, Robert Oania

Labile substrates of the 26S proteasome are earmarked for proteolysis by the covalent attachment of a polyubiquitin (polyUb) chain on acceptor lysines. Our prior work has shown that although the Ub chain is a universal degradation signal, there is specificity in the receptor pathway that is preferentially deployed to target the ubiquitinated protein to the proteasome (Verma *et al.*, 2004). Currently, there are about ten different polyUb binding receptors known in budding yeast. Although all known receptors have a polyUb binding domain (UBD), only a subset of them have a proteasome-binding domain (PBD). The prototype of the latter is Rpn10, which is an intrinsic subunit of the 26S proteasome. Other receptors such as Rad23 and Dsk2 are present in substoichiometric amounts in the 26S proteasome preparations, leading to the "shuttle hypothesis" of delivery of ubiquitinated substrates (reviewed in Elsasser and Finley, 2005). Rpn10 is believed to be the obligate terminal step in this hypothesis. For substrates such as the ubiquitinated Cdk inhibitor Sic1, this may indeed be the case. However, Rpn10 is dispensable for viability. Moreover, Rpn10 is not required for the degradation of substrates such as misfolded CPY* which is degraded at the endoplasmic reticulum (ER). Instead, Cdc48 and its adaptors are needed for degradation of CPY*. The questions that follow from these, and other, observations are: 1) Are there other examples of ubiquitinated substrates that are preferentially recognized by Cdc48 and its adaptors? 2) If yes, are they all localized at the ER, or are there also cytoplasmic and nuclear substrates? 3) How are these ubiquitinated proteins delivered to the 26S proteasome, given that Cdc48 and its adaptors have no bona-fide PBD? 4) How is Rpn10, an intrinsic subunit of the 26S proteasome, bypassed in such pathways?

These are some of the questions that we are attempting to answer in studies on the different receptor pathways of the UPS.

References

Verma *et al.* (2004) *Cell* **118**: 99-110.
Elsasser and Finley (2005) *Nature Cell Biol*. **7**:742-749.

Publications

Cope, G.A. and Deshaies, R.J. (2006) Targeted silencing of JabI /Csn5 in human cells downregulates SCF activity through reduction of F-box protein levels. *BMC Biochem.* **7**:1.

Ghaboosi, N. and Deshaies, R.J. (2007) A conditional yeast E1 mutant blocks the ubiquitin-proteasome pathway and reveals a role for ubiquitin conjugates in targeting Rad23 to the proteasome. *Mol. Biol. Cell* **18**:1953-1963.

Mayor T., Graumann J., Bryan J., Maccoss M.J. and Deshaies R.J. (2007) Quantitative profiling of ubiquitylated proteins reveals proteasome substrates and the substrate repertoire influenced by the Rpn10 receptor pathway. *Mol. Cell Proteomics.* Jul 20; [Epub ahead of print]

Petroski, M.D., Kleiger, G. and Deshaies, R.J. (2006) Evaluation of a diffusion-driven mechanism for substrate ubiquitination by the SCF-Cdc34 ubiquitin ligase complex. *Mol. Cell* **24**:523-534.

Sharon M., Taverner T., Ambroggio X.I., Deshaies R.J., and Robinson C.V. (2006) Structural organization of the 19S proteasome lid: Insights from MS of intact complexes. *PLoS Biol.* Aug;4(8):e267.

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Summary: In eukaryotic cells, the cyclin-dependent kinases (Cdks) control the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The entry into these phases of the cell cycle is controlled by Cdks called S-phase promoting factor (SPF) and M-phase promoting factor (MPF). The action of these Cdks must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms. For example, cells containing unreplicated DNA cannot enter mitosis due to the mobilization of the replication checkpoint. The Dunphy laboratory is engaged in the elucidation of the molecular mechanisms underlying the regulation of SPF and MPF during the cell cycle. Most of these experiments are conducted with *Xenopus* egg extracts, a system in which the entire cell cycle can be reconstituted *in vitro*.

The first member of the cyclin-dependent protein kinase family described is M-phase promoting factor (MPF), which contains the Cdc2 protein kinase and a regulatory subunit known as cyclin B. Since the identification of the molecular components of MPF, there has been rapid and extensive progress in unraveling the biochemistry of mitotic initiation. It is now well established that MPF acts by phosphorylating a myriad of structural and regulatory proteins that are involved directly in mitotic processes such as nuclear membrane disintegration, chromosome condensation, and mitotic spindle assembly. An ongoing challenge to the cell cycle field is the elucidation of how these phosphorylation reactions regulate the structural and functional properties of the various targets of MPF.

We have been most interested in how the cyclin-dependent protein kinases are regulated during the cell cycle. The principal focus of our laboratory has been on the regulatory mechanisms that govern the activation of MPF at the G2/M transition. Some immediate and long-term issues that we are tackling include:

1. What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?
2. How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?
3. What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

More recently, we have been able to study at the molecular level some of the key events leading to the initiation of DNA replication at the G1/S transition. These events involve a cooperative interaction between the Origin Recognition Complex (ORC), the Cdc6 protein, and members of the Mcm family. These studies may ultimately help us understand how S-phase and M-phase are integrated with one another.

In principle, the regulation of cyclin-dependent kinases such as MPF could occur at any of several levels, including synthesis of the cyclin protein, association between the Cdc2 and cyclin proteins, or posttranslational modification of the Cdc2/cyclin complex. The posttranslational regulation of the Cdc2/cyclin complex is particularly important, even in early embryonic cells that manifest the simplest cell cycle programs. In recent years, many of the elaborate details of this Cdc2 modification process have been defined. For example, the binding of cyclin results in three phosphorylations of Cdc2: one at threonine 161 that is required for Cdc2 activity, and two dominantly inhibitory phosphorylations at threonine 14 and tyrosine 15. A variety of genetic and biochemical experiments have established that the inhibitory tyrosine phosphorylation of Cdc2 is an especially important mechanism of cell cycle regulation. As described in greater detail below, there is now strong evidence that the decision to enter mitosis involves considerably more than the tyrosine dephosphorylation of Cdc2. However, a thorough understanding of the kinase/phosphatase network that controls the phosphotyrosine content of Cdc2 will provide a firm foundation for understanding other facets of mitotic regulation.

Our laboratory has made substantial contributions to understanding the molecular mechanisms controlling the activation of the Cdc2 protein. For our studies, we utilize cell-free extracts from *Xenopus* eggs. Due to pioneering work in a number of the laboratories, it is now possible to re-create essentially all of the events of the cell cycle in these extracts. Consequently, it is feasible to study the molecular mechanisms of Cdc2 regulation in intricate detail with this experimental system. To facilitate these studies, we make extensive use of recombinant DNA technology to overproduce cell cycle proteins in either bacteria or baculovirus-infected insect cells. Moreover, in conjunction with our biochemical studies, we are taking advantage of the fission yeast system to exploit genetic approaches to identify novel *Xenopus* regulators of the cell cycle.

419. **Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase**

Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

The checkpoint mediator protein Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing aphidicolin-induced DNA replication blocks. We show that during this checkpoint response Claspin becomes phosphorylated on threonine-906 (T906), which creates a docking site for Plx1, the *Xenopus* Polo-like kinase. This interaction promotes the phosphorylation of Claspin on a nearby serine (S934) by Plx1. After a prolonged interphase arrest, aphidicolin-treated egg extracts typically undergo adaptation and enter into mitosis despite the presence of incompletely replicated DNA. In this process, Claspin dissociates from chromatin and Chk1 undergoes inactivation. By contrast, aphidicolin-treated extracts containing mutants of Claspin with alanine substitutions at positions 906 or 934 (T906A or S934A) are unable to undergo adaptation. Under such adaptation-defective conditions, Claspin accumulates on chromatin at high levels and Chk1 does not decrease in activity. These results indicate that the Plx1-dependent inactivation of Claspin results in termination of a DNA replication checkpoint response.

420. Mcm2 is a direct substrate of ATM and ATR during DNA damage and DNA replication checkpoint responses

Hae Yong Yoo, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

In vertebrates, ATM and ATR are critical regulators of checkpoint responses to damaged and incompletely replicated DNA. These checkpoint responses involve the activation of signaling pathways that inhibit the replication of chromosomes with DNA lesions. In this report, we describe isolation of a cDNA encoding a full-length version of *Xenopus* ATM. Using antibodies against the regulatory domain of ATM, we have identified the essential replication protein Mcm2 as an ATM-binding protein in *Xenopus* egg extracts. *Xenopus* Mcm2 undergoes phosphorylation on serine 92 (S92) in response to the presence of double-stranded DNA breaks or DNA replication blocks in egg extracts. This phosphorylation involves both ATM and ATR, but the relative contribution of each kinase depends upon the checkpoint-inducing DNA signal. Furthermore, both ATM and ATR phosphorylate Mcm2 directly on S92 in cell-free kinase assays. Immunodepletion of both ATM and ATR from egg extracts abrogates the checkpoint response that blocks chromosomal DNA replication in egg extracts containing double-stranded DNA breaks. These experiments indicate that ATM and ATR phosphorylate the functionally critical replication protein Mcm2 during checkpoint responses that prevent the replication of abnormal chromosomes.

421. **Claspin and the activated form of ATR-ATRIP collaborate in the activation of Chk1**

Akiko Kumagai, Soo-Mi Kim, William G. Dunphy

Claspin is necessary for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. ATR possesses a regulatory partner called ATRIP. We have studied the respective roles of ATR-ATRIP and Claspin in the activation of Chk1. ATR-ATRIP binds well to various DNA templates in *Xenopus* egg extracts. ATR-ATRIP bound to a single-stranded DNA template is weakly active. By contrast, the ATR-ATRIP complex on a DNA template containing both single-stranded and double-stranded regions displays a large increase in kinase activity. This observation suggests that ATR-ATRIP normally undergoes activation upon association with specific nucleic acid structures at DNA replication forks. Without Claspin, activated ATR-ATRIP phosphorylates Chk1 weakly in a cell-free reaction. Addition of Claspin to this reaction strongly stimulates the phosphorylation of Chk1 by ATR-ATRIP. Claspin also induces significant autophosphorylation of Chk1 in the absence of ATR-ATRIP. Taken together, these results indicate that the checkpoint-dependent phosphorylation of Chk1 is a multi-step process involving activation of the ATR-ATRIP complex at replication forks and presentation of Chk1 to this complex by Claspin.

422. **Roles of replication fork-interacting and Chk1-activating domains from Claspin in a DNA replication checkpoint response**

Joon Lee, Daniel A. Gold, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

Claspin is essential for the ATR-dependent activation of Chk1 in *Xenopus* egg extracts containing incompletely replicated DNA. Claspin associates with replication forks upon origin unwinding. We show that Claspin contains a replication fork-interacting domain (RFID, residues 265-605) that associates with Cdc45, DNA polymerase epsilon, RPA, and two RFC complexes on chromatin. The RFID contains two basic patches (BP1 and BP2) at amino acids 265-331 and 470-600, respectively. Deletion of either BP1 or BP2 compromises optimal binding of Claspin to chromatin. Absence of BP1 has no effect on the ability of Claspin to mediate activation of Chk1. By contrast, removal of BP2 causes a large reduction in the Chk1-activating potency of Claspin. We also find that Claspin contains a small Chk1-activating domain (CKAD, residues 776-905) that does not bind stably to chromatin, but is fully effective at high concentrations for mediating activation of Chk1. These results indicate that stable retention of Claspin on chromatin is not necessary for activation of Chk1. Instead, our findings suggest that only transient interaction of Claspin with replication forks potentiates its Chk1-activating function. Another implication of this work is that stable binding of Claspin to chromatin may play a role besides the activation of Chk1.

423. **Phosphorylation of Chk1 by ATR in *Xenopus* egg extracts requires binding of ATRIP to ATR but not the stable DNA-binding or coiled-coil domains of ATRIP**

Soo-Mi Kim, Akiko Kumagai, Joon Lee, William G. Dunphy

ATR, a critical regulator of DNA replication and damage checkpoint responses, possesses a binding partner called ATRIP. We have studied the functional properties of *Xenopus* ATR and ATRIP in incubations with purified components and in frog egg extracts. In purified systems, ATRIP associates with DNA in both RPA-dependent and RPA-independent manners, depending on the composition of the template. However, in egg extracts, only the RPA-dependent mode of binding to DNA can be detected. ATRIP adopts an oligomeric state in egg extracts that depends upon binding to ATR. In addition, ATR and ATRIP are mutually dependent on one another for stable binding to DNA in egg extracts. The ATR-dependent oligomerization of ATRIP does not require an intact coiled-coil domain in ATRIP and does not change in the presence of checkpoint-inducing DNA templates. Egg extracts containing a mutant of ATRIP that cannot bind to ATR are defective in the phosphorylation of Chk1. However, extracts containing mutants of ATRIP lacking stable DNA-binding and coiled-coil domains show no reduction in the phosphorylation of Chk1 in response to defined DNA templates. Furthermore, activation of Chk1 does not depend upon RPA under these conditions. These results suggest that ATRIP must associate with ATR in order for ATR to carry out the phosphorylation of Chk1 effectively. However, this function of ATRIP does not involve its ability to mediate the stable binding of ATR to defined checkpoint-inducing DNA templates in egg extracts, does not require an intact coiled-coil domain, and does not depend on RPA.

424. TopBP1 activates the ATR-ATRIP complex

Akiko Kumagai, Joon Lee, Hae Yong Yoo, William G. Dunphy

ATR is a key regulator of checkpoint responses to incompletely replicated and damaged DNA, but the mechanisms underlying control of its kinase activity are unknown. TopBP1, the vertebrate homolog of yeast Cut5/Dpb11, has dual roles in initiation of DNA replication and regulation of checkpoint responses. We show that recombinant TopBP1 induces a large increase in the kinase activity of both *Xenopus* and human ATR. The ATR-activating domain resides in a conserved segment of TopBP1 that is distinct from its numerous BRCT repeats. The isolated ATR-activating domain from TopBP1 induces ectopic activation of ATR-dependent signaling in both *Xenopus* egg extracts and human cells. Furthermore, *Xenopus* egg extracts containing a version of TopBP1 with an inactivating point mutation in the ATR-activating domain are defective in checkpoint regulation. These studies establish that activation of ATR by TopBP1 is a crucial step in the initiation of ATR-dependent signaling processes.

425. Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures

Hae Yong Yoo, Seong-Yun Jeong, William G. Dunphy

The checkpoint mediator protein Claspin is indispensable for the ATR-dependent phosphorylation of Chk1 in response to stalled DNA replication forks in *Xenopus* egg extracts. We show that Claspin also participates in the detection of chromosomal double-stranded DNA breaks (DSBs) in this system. Significantly, removal of Claspin from egg extracts only partially abrogates the activation of Chk1 in response to chromatin with DSBs, whereas depletion of both Claspin and BRCA1 completely abolishes this activation. The function of Claspin in this DSB-triggered pathway depends upon phosphorylation of T817 and S819 by ATR. Conversely, neither phosphorylation of Claspin on these sites nor the presence of BRCA1 is necessary for activation of Chk1 in response to stalled replication forks. Thus, site-specific phosphorylation of a checkpoint mediator protein is a crucial determinant in the discrimination between various checkpoint-inducing structures. Furthermore, checkpoint mediator proteins exhibit functional overlap that varies depending on the nature of the checkpoint-triggering DNA signal.

426. Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM

Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

ATM is necessary for activation of Chk1 by ATR in response to double-stranded DNA breaks (DSBs) but not to DNA replication stress. TopBP1 has been identified as a direct activator of ATR. We show that ATM regulates *Xenopus* TopBP1 by phosphorylating S1131 and thereby strongly enhancing association of TopBP1 with ATR. *Xenopus* egg extracts containing a mutant of TopBP1 that cannot be phosphorylated on S1131 are defective in the ATR-dependent phosphorylation of Chk1 in response to DSBs but not to DNA replication stress. Thus, TopBP1 is critical for the ATM-dependent activation of ATR following production of DSBs in the genome.

427. The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR

Joon Lee, Akiko Kumagai, William G. Dunphy

TopBP1 serves as an activator of the ATR-ATRIP complex in response to the presence of incompletely replicated or damaged DNA. This process involves binding of ATR to the ATR-activating domain of TopBP1, which is located between BRCT domains VI and VII. TopBP1 displays increased binding to ATR-ATRIP in *Xenopus* egg extracts containing checkpoint-inducing DNA templates. We show that an N-terminal region of TopBP1 containing BRCT repeats I-II is essential for this checkpoint-stimulated binding of TopBP1 to ATR-ATRIP. The BRCT I-II region of TopBP1 also binds specifically to the Rad9-Hus1-Rad1 (9-1-1) complex in *Xenopus* egg

extracts. This binding occurs via the C-terminal domain of Rad9 and depends upon phosphorylation of its Ser-373 residue. Egg extracts containing either a mutant of TopBP1 lacking the BRCT I-II repeats or a mutant of Rad9 with an alanine substitution at Ser-373 are defective in checkpoint regulation. Furthermore, an isolated C-terminal fragment from Rad9 is an effective inhibitor of checkpoint signaling in egg extracts. These findings suggest that interaction of the 9-1-1 complex with the BRCT I-II region of TopBP1 is necessary for binding of ATR-ATRIP to the ATR-activating domain of TopBP1 and the ensuing activation of ATR.

Publications

Kumagai, A. and Dunphy, W.G. (2006) How cells activate ATR. *Cell Cycle* **5**:1265-1268.

Kumagai, A., Lee, J., Yoo, H.Y. and Dunphy, W.G. (2006) TopBP1 activates the ATR-ATRIP complex. *Cell* **124**:943-955.

Lee, J., Kumagai, A. and Dunphy, W.G. (2007) The Rad9-Hus1-Rad1 checkpoint clamp regulates interaction of TopBP1 with ATR. *J. Biol. Chem*. **282**:28036-28044.

Margolis, S.S., Perry, J.A., Forester, C.M., Nutt, L.K., Guo, Y., Jardim, M.J., Thomenius, M.J., Freel, C.D., Darbandi, R., Ahn, J.H., Arroyo, J.D., Wang, X.F., Shenolikar, S., Nairn, A.C., Dunphy, W.G., Hahn, W.C., Virshup, D.M. and Kornbluth, S. (2006) Role for PP2A/p56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell* **127**:759-773.

Yoo, H.Y., Jeong, S.-Y. and Dunphy, W.G. (2006) Site-specific phosphorylation of a checkpoint mediator protein controls its responses to different DNA structures. *Genes and Dev*. **20**:772-783.

Yoo, H.Y., Kumagai, A., Shevchenko, A., Shevchenko, A. and Dunphy, W.G. (2007) Ataxia-telangiectasia mutated (ATM)-dependent activation of ATR occurs through phosphorylation of TopBP1 by ATM. *J. Biol. Chem*. **282**:17501-17506.

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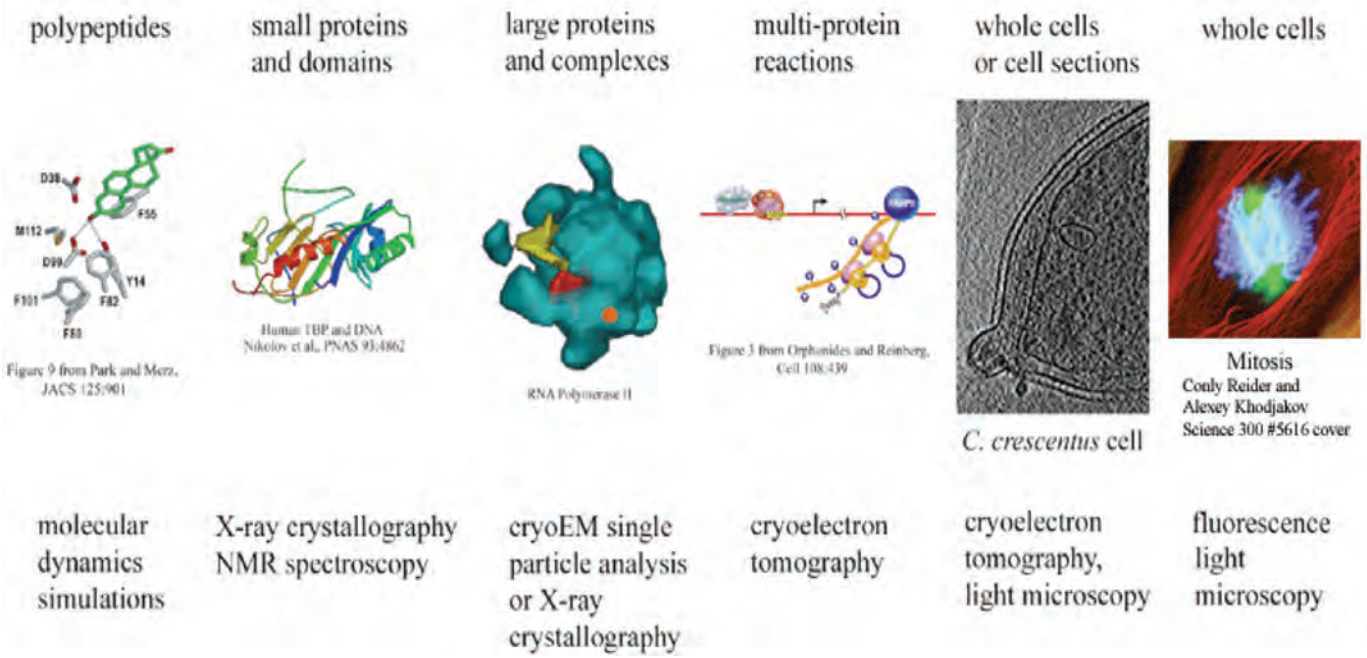
Summary: If we could simply look inside a cell and see its molecular components in all their complexes and conformations, cell biology would be all but finished. We are developing electron-cryomicroscopy-based technologies to do this for at least the largest macromolecular complexes, hoping to show both how individual proteins work together as large "machines" and how those machines are organized into "assembly lines" within living cells. Our electron cryomicroscopy projects range from imaging individual macromolecules to larger protein assemblies to viruses to organelles and even to intact cells. In addition, we have now begun simulation projects to understand how such spatial relationships affect cellular processes.

We use two basic data collection strategies. The first, called "tomography," involves imaging a single unique object (such as a cell) from multiple directions and then merging those projections into a three-dimensional reconstruction. The second, called "single particle analysis," involves imaging a large number of identical copies of a target object (such as a purifiable protein complex), and again merging the images to produce a three-dimensional reconstruction. Both techniques are still rapidly developing, but together hold the promise of completing what could be called the "structural biology continuum" (**Figure 1**) in a step-wise fashion by showing first how individual proteins (visible by X-ray crystallography and NMR spectroscopy) come together to form complexes (visible by single particle analysis), and how those complexes are organized within living cells (visible by electron tomography). This structural data, in turn, will inform whole-cell models of the interactions of all the cell's molecular components.

Both single particle analysis and tomography begin by spreading the sample in a thin film (~300 nm) across an electron microscope grid and then plunging it into liquid ethane, which causes the water to form vitreous, rather than crystalline, ice, and preserves the sample in a native state without any unnatural fixatives, resins, or stains. For tomography, the sample is imaged from a series of angles by incrementally tilting a goniometer through ~140 degrees. For single particle analysis, each copy of the sample (one "particle") freezes with a random orientation with respect to the plane of the grid so that tilting is not necessary. Instead, images of hundreds of thousands of individual particles are recorded and then aligned and averaged in three-dimensions computationally. The target resolution for single particle analysis is 4-10Å, where the secondary structure of a particle can be seen and fitting atomic models of components is possible. The target resolution for tomography is 3-6 nm, where the identity, location, and orientation of individual macromolecules can be seen in their cellular contexts.

Our work involves technology development as well as its application to key biological questions. We enjoy the use of a state-of-the-art, 300 kV, helium-cooled, energy-filtered, fully automated, dual-axis tilting, FEG "Polara" TEM. This year's technical efforts were directed towards: (1) simulation studies to understand the effect of alignment errors in single particle reconstructions; (2) theoretical work on methods to correct for curvature of the Ewald sphere; and (3) developing software to automate tilt-series acquisition. Some of the biological questions we have addressed this year are: (1) the nature of the prokaryotic cytoskeleton, (2) the structure of the bacterial flagellar motor; (3) the structure of immature HIV-1 virus-like particles, (4) the quaternary structure of various RNA polymerase complexes, (5) the structure of the bacterial carboxysome, and (6) the ultrastructure of the smallest known eukaryotic cell.

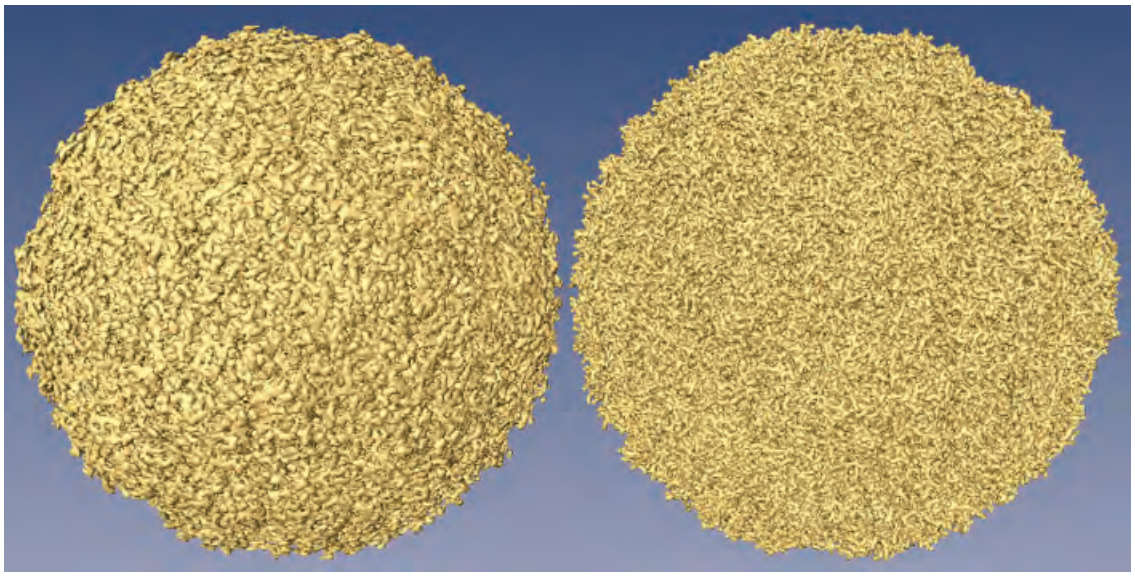


428. Ewald sphere correction for large icosahedral virus particle reconstruction

Peter W. K. Leong, Grant J. Jensen

When an electron microscope records an image of an object, information from the Fourier values on the Ewald sphere contribute to form an image. The usual approximation made is that the image is a perfect projection of the object and instead contains information from a central slice of the 3D Fourier transform of the object. However, the approximation begins to fail at high resolutions, low acceleration voltages and for large virus particles because under these conditions, the information on the Ewald sphere becomes significantly different from the information found on a central slice, thereby limiting the maximum resolution of the 3D reconstruction of the object.

We have developed a reconstruction algorithm to take into account the curvature of the Ewald sphere and are implementing it in two different image-processing packages. Our simulations have shown that the resolution limit, which is a result of the central slice approximation, can be corrected for, thereby improving the resolution of 3D reconstructions. The algorithm is also being applied to an experimentally-obtained dataset of a large virus particle to confirm the resolution improvements on real data.



4Å (left) and 2Å (right) reconstructions of Foot and Mouth Virus capsid using Ewald-sphere-simulated-images illustrate the improvement in resolution when taking the Ewald sphere into account.

429. Role of the carboxysome in microbial carbon fixation

Dylan Morris, Grant J. Jensen

The goal of this project is to uncover the mechanism of carboxysome function in microbial carbon fixation. Our hypothesis is that the protein shell of the carboxysome provides a selectively permeable barrier to substrate diffusion and thereby increases the catalytic efficiency of the rate-limiting enzyme in carbon fixation, ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCO).

Carboxysomes are icosahedral inclusionary bodies found in many chemoautotrophic bacteria and in all cyanobacteria. They represent the terminal stage of the CO_2 concentrating mechanism (CCM) of autotrophic bacteria and are produced in greater number when organisms are grown at limiting CO_2 concentrations. Carboxysomes are composed entirely of protein and range in diameter from 100-400 nm. They enclose approximately 250 copies of the RuBisCO holoenzyme within a 4 nm-thick protein shell.

RuBisCO catalyzes the fixation of CO_2 to the five carbon sugar, ribulose-1,5-bisphosphate, in the first step of the Calvin cycle. RuBisCO also catalyzes the fixation of O_2 in an unproductive and energy-requiring photorespiration reaction. RuBisCOs of different organisms are therefore characterized by a "specificity factor," which relates their rate of carboxylation to their rate of oxygenation. All experimental evidence demonstrates that the segregation of RuBisCOs into the carboxysome offers a catalytic advantage to the enzyme, however, the precise mechanism by which this occurs has yet to be determined.

We are currently performing molecular dynamics simulations of the carboxysome shell proteins. From these simulations, we will determine the permeability of the shell to the various substrates of the RuBisCO enzyme. Once we have these values, we will model the carboxysome system at the Smoluchowski level of abstraction within the cellular geometries acquired from our tomograms. These simulations will yield predictions for the expected carbon fixation rates of the cells. We will compare these predictions to the observed fixation rates and see whether our assumptions regarding selective permeability are sufficient to explain the data.

430. Image processing software tool development and applications

H. Jane Ding, Grant J. Jensen

Software development in image processing plays crucial roles in electron microscopy. We employ existing software packages as well as writing our own as needed for reconstructions and structural analysis. We have been developing various techniques in tomogram analysis for obtaining structural information of the molecular complexes through electron microscopy. Below are some of the main topics we are currently working or planning to work on:

1. Tomogram segmentation: Manual segmentation of tomograms is often subjective and can be tedious. It is one of our goals to develop reliable quantitative approaches for interpretation of features in tomograms, either automatic or semi-automatic. Various methods are being developed for approaching this goal.
2. Analysis and visualization: Visualization is one of the most effective ways to understand the targeted structures. We have developed/are developing various programs that enable users to compute and/or view certain features in 3D images.
3. Pattern recognition and analysis: Pattern recognition methods can be used for a systematic interpretation of the tomograms. Our research targets pattern recognitions in 3D as well as 2D (on surfaces). Methods for detecting and averaging "unit cells" of interest from ordered regions are being developed.
4. Tomogram analysis is challenging because of the low signal-to-noise ratios of the EM images as well as the "missing wedge" in tomography. Our future work is also aimed at improving the quality of the images, which includes implementing new software or improving the software that is currently used in three-dimensional reconstruction tomography.

431. Fluorescent light microscopy and correlated electron tomography of the *Caulobacter crescentus* cytoskeleton

Ariane Briegel, D. Prabha Dias, Matt Cabeen[1], Christine Jacobs-Wagner[1], Rasmus Jensen[2], Grant J. Jensen

Until relatively recently, it was thought that bacteria had very little regular internal structure and that they were essentially a "bag of macromolecules." Mounting evidence that this is not the case includes the discovery of FtsZ and MreB, the bacterial homologs to the eukaryotic cytoskeletal proteins tubulin and actin, respectively, and the visualization by electron-cryo-tomography (ECT) of filaments in bacteria. The sequencing of the *C. crescentus* genome revealed the presence of cresentin, a homolog to a third type of cytoskeletal element, intermediate filaments. This bacterium is thus, an excellent place to look for a bacterial version of the eukaryotic cytoskeleton.

Four distinct types of filament bundles were observed in tomograms of wild-type *C. crescentus* cells and were named according to their shape and localization: 'inner curvature,' 'polar,' 'cytoplasmic' and 'ring-like' bundles. In order to reveal the identity of these bundles, as well as to visualize the known cytoskeletal elements like MreB and Crescentin, we are using correlated fluorescence light microscopy (FLM) and ECT.

Since non-invasive intracellular labeling techniques for cryo-ET are not presently available, ECT can be combined with fluorescent light microscopy (FLM). This allows one to pair widely used fluorescent labeling methods with the high resolution of electron tomography. So far, we have succeeded in imaging the same individual

C. crescentus cells with GFP-labeled Crescentin by FLM and ECT. Combined with computational searches, this approach will help us to reveal the localization patterns, appearance and function of these cytoskeletal elements.

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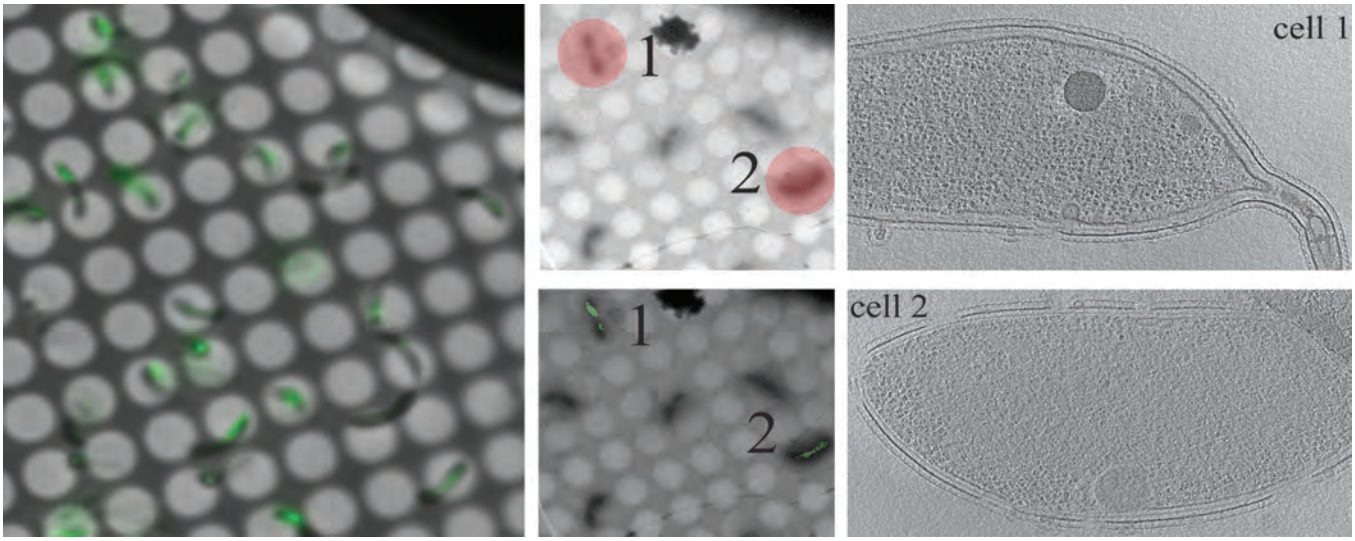

C. crescentus cells with GFP-labeled Crescentin (strain CJW 815) are immobilized with Poly-L-Lysine on finder grids and observed by FLM (left). After plunge-freezing the sample in liquid ethane, the same individual cells can be identified in the electron microscope (upper middle) and correlated to FLM images. Tomography (right) of these cells enables us to combine the high resolution of the electron microscope, the three-dimensionality of tomography and the molecular labeling of the FLM.

432. 3-D ultrastructure of *Ostreococcus tauri*: Electron cryotomography of an entire eukaryotic cell

Gregory Henderson, Grant J. Jensen

The hallmark of eukaryotic cells is their segregation of key biological functions into discrete, membrane-bound organelles. Creating accurate models of their ultrastructural complexity has been difficult in part because of the limited resolution of light microscopy and the artifact-prone nature of conventional electron microscopy. Here we explored the potential of the emerging technology electron cryotomography to produce 3-D images of an entire eukaryotic cell in a near-native state. *Ostreococcus tauri* was chosen as the specimen because as a unicellular picoplankton with just one copy of each organelle, it is the smallest known eukaryote and was therefore likely to yield the highest resolution images. Whole cells were imaged at various stages of the cell cycle, yielding 3-D reconstructions of complete chloroplasts, mitochondria, endoplasmic reticula, Golgi bodies, peroxisomes, microtubules, and putative ribosome distributions *in situ*. Surprisingly, the nucleus was seen to open long before mitosis, and while one microtubule (or two in some predivisional cells) were consistently present, no mitotic spindle was ever observed, prompting speculation that a single microtubule might be sufficient to segregate multiple chromosomes.

433. Electron cryotomography of HIV-1

Elizabeth R. Wright, Jordan Benjamin, H. Jane Ding, Grant J. Jensen

The major structural elements of retroviruses are contained in a single polyprotein, Gag, which in HIV-1 comprises the MA, CA, SP1, NC, SP2, and p6 proteins. In the immature HIV-1 virion, the domains of Gag are arranged radially with the amino-terminus of MA at the membrane and NC-p6 facing the particle center. During maturation, viral protease (PR) cleaves Gag between the major domains (MA, CA, and NC). In the mature virion, MA remains bound to the membrane, while the processed CA subunits form a central conical capsid that encases NC, the RNA genome, and other viral enzymes. While all HIV-1 virions assemble with similar architectures using the same basic polyprotein building blocks, they have different sizes, numbers of Gag molecules, and ultrastructures. The unique nature of each virion has meant that crystallographic techniques could not be applied to examine intact virions. Therefore, our present understanding of HIV-1 organization is from conventional electron microscopy. The advent of electron cryotomography (cryo-ET) has provided us with a method by which we are able to obtain full 3-D reconstructions of individual, asymmetric objects such as HIV-1.

We have analyzed the ultrastructure of both the immature and mature forms of HIV-1 via cryo-ET (Benjamin *et al*., 2005; Wright *et al*., 2007). Our current research is divided into two main areas as outlined below.

1. Placement and structure of Env on HIV-1 virions: The Env protein complex, which protrudes from the viral surface, is necessary for viral infectivity. We are currently imaging immature and mature HIV-1 virions expressing the envelope (Env) proteins (Gp120 and Gp41) to determine the placement and structure of Env on the virions throughout maturation (**Figure 1**).
2. Correlated fluorescence light microscopy (fLM) and cryo-ET: We have begun to examine the virions within the cellular context by correlating observations made by fLM and cryo-EM methods (**Figure 2**).

Reference

Wright, E.R*, Schooler, J.B.*, Ding, H.J., Kieffer, C., Fillmore, C., Sundquist, W.I. and Jensen, G.J. (2007) *EMBO J.* **26**:2218.

**These authors contributed equally.*

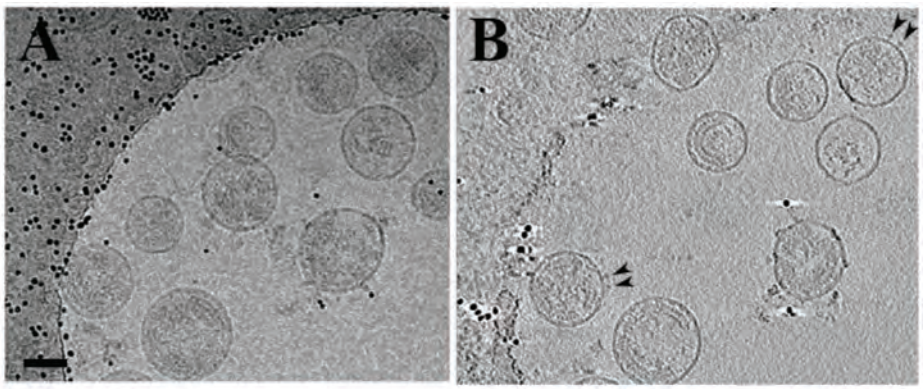

Figure 1. *Example raw image and reconstruction.* The untilted image from a tilt series of isolated immature HIV-1 virions suspended in vitreous ice (left). Slice (5.6 nm) through the middle of the resulting 3D reconstruction, showing the visibly ordered Gag lattice below the membrane and Env protruding outward from the membrane (arrowheads). Scale bar 50 nm.

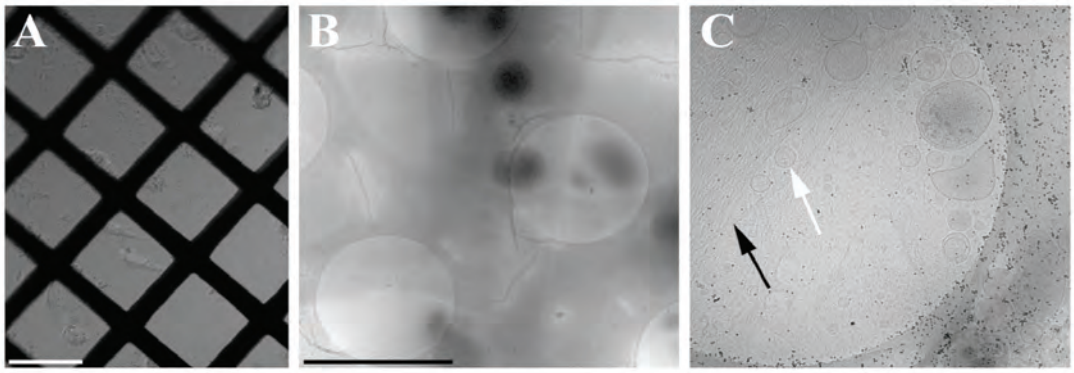

Figure 2. *COS cells immobilized on an EM grid.* LM image of COS cells on an EM grid, scale bar 200 μm (left). Cryo-EM image of COS cells in which cellular appendages have spread across the grid and contain microtubule bundles and small and large vesicles (middle). Higher magnification cryo-EM image of the cellular cytoplasm, note the networks of microtubules (black arrow) and a vesicle (white arrow) approximately the size of an HIV-1 virion.

434. Structure of Vps4 and its Vta1 complex by electron cryomicroscopy

Zhiheng Yu, Malgorzata D. Gonciarz, Wesley I. Sundquist*, Christopher P. Hill*, Grant J. Jensen*

The budding of retroviruses from cell membranes and the budding of intracellular vesicles into the multivesicular body utilize a common class of cellular machinery called the 'Class E' vacuolar protein sorting (Vps) proteins. Most Class E proteins are involved in the formation of three endosomal sorting complexes required for transport (ESCRT) that are successively recruited to the sites of vesicle formation to function in cargo recognition, protein sorting and membrane deformation and fission. The budding machinery is reset by Vps4, a type-I AAA ATPase, that is thought to drive disassembly and recycling of ESCRT complexes. Vps4 imparts directionality to the budding process and is required for multiple rounds of vesicle formation. It may also play a

direct role in protein sorting and membrane fission. The budding of all retroviruses tested to date, including HIV, also requires Vps4.

In an effort to better understand the structure and function of Vps4 in particular and AAA proteins in general, we imaged three different Vps4 oligomeric complexes with and without their N-terminal domains and Vta1 co-factors by electron cryomicroscopy (cryo-EM). Vps4 is shown to form a dodecameric bowl-like structure composed of two stacked hexameric rings that adopt dramatically different conformations. Consistent with their role in substrate binding, the N-terminal domains of the top, open ring localize on the symmetry axis above the presumed active site in the bowl's central cavity. The N domains of the bottom, closed ring localize around the periphery in the form of six fins. Consistent with its roles in both substrate binding and complex stabilization, Vta1 appears to bind in multiple locations including above the bowl's central cavity, on the periphery of the bottom ring where Vps4's β-domain likely sits, and underneath the bottom ring. By comparing these structures with other AAA proteins we propose that Vps4 might bind substrates in its large central chamber in the course of removing the ESCRT-III subunits from their membrane-bound lattice.

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Figure. 1: Single particle cryo-EM reconstruction of Vta1-Vps4 complex.

435. Electron cryotomography of purified carboxysomes

Cristina V. Iancu, H. Jane Ding, Dylan M. Morris, Grant J. Jensen

Carboxysomes are organelle-like polyhedral bodies enclosed by a proteinaceous shell and filled with ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) molecules, a key enzyme in CO_2 fixation. They are part of the CO_2 concentrating machinery (CCM) in cyanobacteria and many chemoautotrophic bacteria, and are produced in a greater number when the organisms are grown under low-CO_2 conditions. Exactly how carboxysomes help the organisms compete when CO_2 is limited is still unclear. We employ electron cryotomography to explore the 3D structure of purified carboxysomes. These studies will provide a more detailed view of these organelles allowing us to determine their overall shape, the number and organization of RuBisCOs thus, leading to a better understanding of the structure-function relationship.

We have looked at carboxysomes purified from two different sources: the *Synechococcus* strain WH8102 and *Halothiobacillus neapolitanus (H. neapolitanus)*. Both kinds of carboxysomes vary in size and they have an icosahedral overall shape that seems more regular for *Synechococcus*. RuBisCO molecules are organized in shells spaced about 12 nm apart. Simulations of carboxysomes in which RuBisCO molecules are randomly placed revealed that, as the number of enzymes increased, shells spontaneously arise similarly to the ones in the data. So far the resolution of the data is insufficient to support specific interactions between RuBisCOs. Recent studies on the shell proteins showed that two of these proteins form charged pores that probably provide charged metabolites to the RuBisCOs, and another shell protein is a novel carbonic anhydrase supposed to concentrate CO_2 proximally to RuBisCOs. Thus, it appears that the carboxysome shell acts both as a permeable membrane for substrates and as a template, organizing RuBisCO in specific patterns that optimize CO_2 fixation.

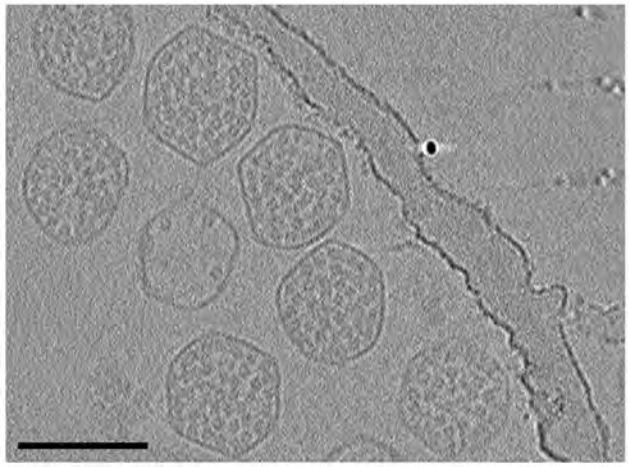

Figure: Slice through a tomogram of purified *H. neapolitanus* carboxysomes. Scale bar corresponds to 150 nm.

436. Electron cryotomography of *in situ* carboxysomes

Cristina V. Iancu, Grant J. Jensen

In parallel with the studies of purified carboxysomes, we also examine carboxysomes within cells to ensure that the purified material is representative of the *in vivo* carboxysomes and to establish what cellular ultra-structural elements are associated with them. A long-term goal is to capture different stages of carboxysome assembly within cells.

As *Synechococcus* cells are too large to be amenable to electron cryotomography studies, we chose *H. neapolitanus* cells for visualizing carboxysomes *in situ*. Tomograms of these cells typically show about seven carboxysomes/cell. Most cellular carboxysomes are similar in size and shape to the purified ones, however, there are also some unusually long. There are no visible

filaments linking the carboxysomes, they just seem to be dispersed randomly in the cytoplasm of the cell. The most surprising finding is the existence of a high-density aggregate of variable size within the majority of the cellular carboxysomes. As this element has never been seen in the purified carboxysomes we speculated that it might be the result of accumulated metabolites that have not been released yet from carboxysomes and that the purification process would dilute them away. We are in the process of testing this hypothesis by looking at cells that are slowed down metabolically.

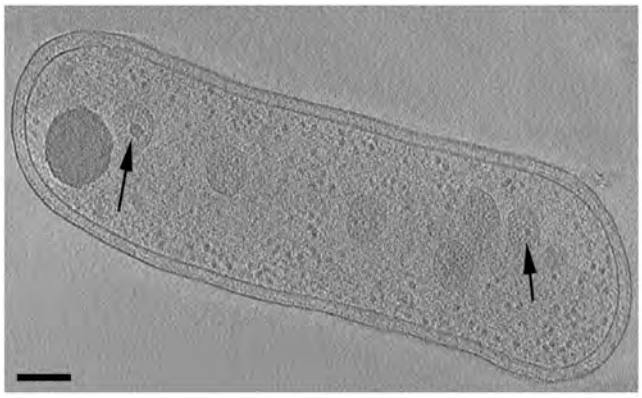

Figure: Slice through a tomogram of an *H. neapolitanus* cell. Scale bar corresponds to 150 nm. Arrows indicate the high-density aggregates present in cellular carboxysomes.

Publications

Briegel, A., Dias, D.P., Li, Z., Jensen, R.B., Frangakis, A.S. and Jensen, G.J. (2006) Multiple large filament bundles observed in *Caulobacter crescentus* by electron cryotomography. *Mol. Microbiol*. **62**(1):5-14.

Burns, D.G, Janssen, P.H., Itoh, T., Kamekura, M., Li, Z., Jensen, G.J., Rodriguez-Valera, F., Bolhuis, H. and Dyall-Smith, M.L. (2007) Haloquadratum walsbyi gen. nov., sp. nov., the square haloarchaeon of Walsby, isolated from saltern crystallizers in Australia and Spain. *Int. J. Syst. Evol. Microbiol*. **57**:387-392.

Henderson, G.P. and Jensen, G.J. (2006) Three-dimensional structure of *Mycoplasma pneumoniae*'s attachment organelle and a model for its role in gliding motility. *Mol. Microbiol*. **60**(2):376-385.

Iancu, C.V., Ding, H.J., Morris, D.M., Martino, A. and Jensen, G.J. (2007) The structure of isolated *Synechococcus* strain WH8102 carboxysomes as revealed by electron cryotomography. *J. Mol. Biol.* In press.

Iancu, C.V., Tivol, W.F., Schooler, J.B., Henderson, G.P., Dias, D.P., Murphy, G.E., Wright, E.R., Li, Z., Briegel, A., Yu, Z., Gan, L., He, Y. and Jensen, G.J. (2006) Electron cryotomography sample preparation. *Nature Protocols*, DOI 10.1038/nprot.2006.432.

Iancu, C.V., Wright, E.R., Heymann, J.B. and Jensen, G.J. (2006) A comparison of liquid nitrogen and liquid helium as potential cryogens for electron cryotomography. *J. Struct. Biol.* **153**:231-240.

Jensen, G.J. (2007) A nanoengine for gliding motility (Invited microcommentary). *Mol. Microbiol*. **63**:4-6.

Jensen, G.J. and Briegel, A. (2007) How electron cryotomography is opening a new window into prokaryotic ultrastructure. *Curr. Opin. Struct. Biol*. **17**:260-267.

Komeili, A., Li, Z., Newman, D.K. and Jensen, G.J. (2006) Magnetosomes are cell membrane invaginations organized by the actin-like protein MamK. *Science* **311**:242-245.

Murphy, G.E., Leadbetter, J.R. and Jensen, G.J. (2006) *In-situ* structure of the complete *Treponema primitia* flagellar motor. *Nature* **442**:1062-1064.

Wright, E.R., Iancu, C.V., Tivol, W.F. and Jensen, G.J. (2006) Observations on the behavior of vitreous ice at ~82 and ~12 K. *J. Struct. Biol*. **153**:241-252.

Wright, E.R., Schooler, J.B., Ding, H.J., Kieffer, C., Fillmore, C., Sundquist, W.I. and Jensen, G.J. (2007) Electron cryotomography of immature HIV-1 virions reveals the structure of the CA and SP1 Gag shells. *EMBO J.* **26**:2218-2226.

Professor: Stephen L. Mayo
Research Fellows: Roberto Chica, Eun Jung Choi, Karin Crowhurst, Alex M. Perryman, Corey J. Wilson
Graduate Students: Benjamin D. Allen, Oscar Alvizo, Jennifer R. Keeffe, J. Kyle Lassila, Heidi K. Privett, Christina Vizcarra
Research and Laboratory Staff: Marie L. Ary, Rhonda K. Digiusto, Cathy J. Miles
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Summary: The focus of the lab has been the coupling of theoretical, computational, and experimental approaches for the study of structural biology. In particular, we have placed a major emphasis on developing quantitative methods for protein design with the goal of developing a fully systematic design strategy that we call "protein design automation." Our design approach has been captured in a suite of software programs called ORBIT (Optimization of Rotamers By Iterative Techniques) and has been applied to a variety of problems ranging from protein fold stabilization to enzyme design.

437. Testing energy functions for computational protein design using a weakest-link screen

Benjamin D. Allen[*]*, Corey J. Wilson, Christina Vizcarra*[*]*, Stephen L. Mayo*

Computational protein design algorithms must sift through an astronomical number of amino acid sequences to find a small subset expected to stabilize the target fold and perform some function. Many approximations are required to make this type of calculation feasible; most notably, the coordinates of the main chain are held fixed, and solvent molecules are not explicitly modeled. These simplifications necessitate the use of heuristic, data-driven, and negative-design potential functions to boost the predictive power of these calculations to acceptable levels. We have recently incorporated two such potential functions into our design procedure. The first is a phi/psi propensity term that benefits or penalizes amino acids at each site based on the main-chain conformation. The energies are derived from the analysis of a high-resolution structural database. The second is a continuum model of polar and nonpolar desolvation that is based on an occlusion model and parameterized using the structural database and high-level Poisson-Boltzmann solvation calculations. These terms contain nonthermodynamic negative-design components, making the process of assigning scale factors to each term nontrivial. We have chosen to assess the utility of these new energy functions directly by using them to design libraries of adenylate kinase (ADK). The libraries will be screened using a strain of the thermophilic organism *Thermatoga neapolitana* in which the wild-type thermophilic ADK has been replaced with a mesophilic homolog. In this system, the organism will only survive above the denaturation temperature of the mesophilic ADK if the designed ADK is folded and active. We plan to screen libraries of designed ADK variants at several temperatures and exhaustively sequence them, leading to an extensive dataset that will allow us to compare the performance of the new energy functions to current methodology. The energy functions can also be re-weighted using the data generated by these experiments, and the resulting weights can be assessed by using them to design additional libraries for testing.

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438. Modifying the spectral properties of a red fluorescent protein

Roberto A. Chica, Benjamin D. Allen[*]*, Stephen L. Mayo*

Fluorescent proteins have found widespread application in cell biology as reporters for the cellular localization of various proteins. In the last decade, many fluorescent proteins with emission spectra spanning the entire visible region have been isolated in nature or have been developed in the laboratory. Those that emit in the red portion of the visible spectrum are of particular interest, because longer wavelengths are less toxic to cells, and because cells are more transparent to red light. DsRed, a tetrameric red fluorescent protein found in the sea anemone *Discosoma striata*, has been extensively studied and has been evolved to yield monomeric red fluorescent proteins with red-shifted emission maxima. One such DsRed mutant, mCherry, has a very red-shifted emission maximum of 610 nm. Our goal is to further evolve mCherry to yield mutants with improved spectral properties, such as an even more red-shifted emission maximum, higher quantum yield, and increased brightness. We will use a computational design method to generate "smart" combinatorial libraries of mCherry. The protein sequences in these libraries will have a higher probability of folding properly, since our method eliminates mutations that would destabilize the protein fold. The designed library will include the Ile197Tyr mutation, which is expected to red shift the emission maximum by allowing a π-stacking interaction with the chromophore. Six residues surrounding Tyr197 will be designed to stabilize the Ile197Tyr mutation. This will yield a library of ~500 mutants, a size that can easily be screened for the desired properties using a 96-well plate-based assay. In addition, exhaustive sequencing of all the fluorescent mutants from the library will provide information on structure/function relationships and will facilitate optimization of the computational method.

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439. **Using negative design to increase HIV protease specificity**

Oscar Alvizo[*], *Stephen L. Mayo*

HIV protease is essential for viral maturation. Its main role is to recognize and cleave sequences in the Gag and Pol polyproteins. The enzyme is of unique interest because its symmetrical binding region recognizes and cleaves asymmetrical substrates. In addition, its natural substrates exhibit little sequence homology (three are shown in the table below). Our goal is to exploit HIV protease's asymmetric substrate recognition by designing hetero-dimer mutants with increased specificity for one of three natural substrates, CA-p2, p2-NC or RT-RH. Crystal structures are available for an inactive variant of HIV protease bound to each of these substrates; these complexes serve as scaffolds for computational protein design.

The optimization procedure uses all three scaffolds simultaneously and employs a scoring function chosen to predict mutations that will stabilize one scaffold over the other two. To insure viable sequences, only sequences with energies within 20% of the global minimum energy conformation (GMEC) of the positive design structure are evaluated. Within this restraint, the search algorithm tries to recover sequences with detrimental interactions in the two negative design structures. The predicted mutants are visually inspected and promising mutations are selected for experimental validation.

This type of negative design procedure proved to be the most effective in producing desirable mutants. To date, three mutants, one for each of the substrates, have been selected and experimentally tested. Preliminary data suggest that each of these mutants exhibits increased specificity towards its intended substrate. More rigorous testing is currently underway to quantitate this effect.

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Substrate	P5	P4	P3	P2	P1	P1´	P2´	P3´	P4´	P5´
CA-p2	Lys	Ala	Arg	Val	Leu	Ala	Glu	Ala	Met	Ser
p2-NC	Pro	Ala	Thr	Ile	Met	Met	Gln	Arg	Gly	Asn
RT-RH	Gly	Ala	Glu	Thr	Phe	Tyr	Val	Asp	Gly	Ala

440. **Probing the mechanism of the ligand-induced decrease in fluorescence in an LTP biosensor**

Eun Jung Choi, Stephen L. Mayo

Recently, we used computational protein design to modify lipid transfer protein (LTP) so that it could act as a biosensor [1]. One cysteine from one of the four native disulfide bonds was used to attach acrylodan, an environmentally sensitive thiol-reactive fluorophore. The other cysteine was eliminated and interactions were computationally designed in its place. When titrated with palmitate, a native ligand of LTP, fluorescence emission decreased; this decrease was used to obtain the binding affinities of LTP and the engineered variants.

Variants that utilized the C4-C52 disulfide bond for acrylodan attachment showed the most marked difference in fluorescence signal upon ligand binding. We propose that the removal of the C4-C52 disulfide bridge provides the N-terminal helix flexibility and allows acrylodan to insert into the binding pocket. Upon ligand binding, acrylodan may be displaced from an ordered nonpolar environment to a disordered polar environment, causing the fluorescence decrease. Several results support this mechanism. The apo-protein stabilities are increased, while the stabilities of the ligand-bound proteins are decreased compared to the acrylodan-free variants. Additional support is provided by the observed decrease in fluorescence emission and the red shift of the emission maximum as palmitate is added.

To test this proposed mechanism, we constructed the C4H/C52A/N55E variant with a Cys tether. We assumed that if acrylodan were attached to the Cys on a flexible tether rather than proximate to the hydrophobic pocket, the change in fluorescence would be greater. A GGSGCGS tether was attached to the C-terminus of C4H/C52A/N55E. Unfortunately, the T_m data did not show the stabilization of the apo form and destabilization of the ligand-bound form (compared to untethered C4H/C52A/N55E) that we anticipated. The linker may not have been long/flexible enough. Also, the linker was attached to the C-terminus for cloning reasons, but it might be critical to attach it to the N-terminus, near the disulfide. In addition, we compared the T_m of the tethered acrylodan-conjugated variant with that of the untethered acrylodan-free variant. The tether might destabilize the protein; therefore, the tethered acrylodan-conjugated variant should be compared with the tethered acrylodan-free variant.

Reference

[1] Choi, E. J., Mao, J. and Mayo, S.L. (2007) *Protein Sci.* **16**:582-588.

441. **Structural and dynamic analysis of computationally designed protein G variants**

Karin Crowhurst, Stephen L. Mayo

Protein function is frequently reliant on dynamic processes; successful protein-protein interactions and enzyme catalysis often require structural flexibility and plasticity. These entropic factors can also contribute to protein stability. Understanding protein dynamics in computationally designed proteins compared to wild type could therefore be crucial for improving the accuracy and consistency of stable *de novo* protein and enzyme production, with wide-ranging medical and industrial applications. My project goal has been to understand the degree to which our current protein design algorithm (ORBIT) produces proteins with native-like backbone dynamics; information learned can be used to target future algorithm modifications and to move towards deliberately incorporating backbone flexibility into designs.

NMR spectroscopy has been employed for detailed analysis of the backbone dynamics of several mutants of streptococcal protein Gβ1; the structural flexibility of these mutants is believed to significantly impact their respective stabilities compared to wild type [1]. Previously, we reported that a highly thermostable variant (Gβ1-c3b4) [2] dimerizes in solution; our 2.3 Å crystal structure shows that the interface is likely formed through a β-bridging interaction with antiparallel β2

strands. NMR relaxation experiments have revealed significant conformational exchange for all backbone amides in the β2 strand and the adjacent region of the helix. In contrast, the backbone amides involved in intermediate timescale motions in the six-fold core mutant (FILIIW) are localized in residues close to two mutations (at either end of the helix). There is also evidence for weak self-association in this variant at high concentrations. Dynamics in both variants may be attributed to side chain overpacking or the loss of specific interactions (such as hydrogen bonds or aromatic ring stacking, which rigidify the core of FILIIW). However, increased motion is due primarily to new non-specific interactions created by greater hydrophobic burial in the variants, which would lead to higher overall entropy (and therefore, altered stabilities) compared to wild type. These data provide insight into the properties of protein sequences generated by ORBIT according to specific design criteria and highlight aspects of the algorithm that may require modification to produce variants with desired dynamic characteristics.

References

[1] Su, A. and Mayo, S.L. (1997) *Protein Sci.* **6**:1701-1707.
[2] Malakauskas, S.M. and Mayo, S.L. (1998) *Nature Struct. Biol.* **5**:470-475.

442. Designing calmodulin specificity: Incorporation of explicit negative design

Jennifer R. Keeffe[*]*, Stephen L. Mayo*

Protein-protein interactions are essential to many processes within cells, including signaling pathways, the immune response, and in the formation of the functional, oligomeric state of many proteins. The ability to design a protein with high affinity and specificity to a particular protein or peptide target would not only increase our understanding of protein-protein interactions, but also aid in the generation of new therapeutics, diagnostics and research tools.

Calmodulin (CaM) is an ideal system for studying the specificity of protein-protein interactions by computational design. CaM is a small Ca^{2+} binding protein that binds to and regulates numerous proteins *in vivo* with high affinity, including smooth muscle myosin light chain kinase (smMLCK) and CaM kinase I (CaMKI). In addition, several high-resolution CaM-peptide complexes have been solved by X-ray crystallography.

Previously, the ORBIT computational design software was successfully used to generate a CaM variant with increased specificity toward the smMLCK peptide [1]. However, this variant did not discriminate between the smMLCK and CaMKI peptides [2], indicating that there is potential for improvement by including a negative design component against the CaMKI target.

Our current studies are focused on designing a CaM variant that can efficiently discriminate between the smMLCK peptide and the CaMKI peptide by incorporating explicit negative design. Toward this goal, we are using ORBIT to optimize CaM side chains that interact with the peptides. Using an optimization algorithm written by graduate student Benjamin Allen, the side chains are simultaneously optimized using positive design against the CaM-smMLCK peptide structure and negative design against the CaM-CaMKI peptide structure. Currently, we have assayed many designed CaM variants using tryptophan fluorescence to assess peptide binding. All variants are properly folded and retain the ability to bind peptides. Initial studies indicate that one variant exhibits a 10-fold preference for the smMLCK peptide over the CaMKI peptide. Future experimental validation of these and other computationally designed variants will include Biacore binding assays to determine the binding affinity of CaM to the peptides. We are also investigating multiple negative design scoring functions to obtain variants that are experimentally verified to show changes in specificity.

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References

[1] Shifman, J.M. and Mayo, S.L. (2002) *J. Mol. Biol.* **323**:416-423.
[2] Shifman, J.M. and Mayo, S.L. (2003) *Proc. Natl. Acad. Sci. USA* **100**:13274-13279.

443. Importance of secondary active site residues in *E. coli* chorismate mutase

Jonathan Kyle Lassila[1]*, Jennifer R. Keefe*[1]*, Peter Kast*[2]*, Stephen L. Mayo*

The success of computational enzyme design efforts is limited by our understanding of the structural determinants of catalysis and our ability to model these factors computationally. To investigate the distribution of active enzyme catalysts in sequence space and to evaluate present computational design procedures, we measured the effects on stability and catalysis of 114 single mutations to secondary active site residues of *Escherichia coli* chorismate mutase. All 19 amino acids were tested in six active site positions that do not directly contact the transition state analog in the enzyme crystal structure. The six positions were chosen to clarify the results of a previous computational enzyme design experiment. Each of the 114 variants was tested for its ability to promote viability in a chorismate mutase deletion strain, which cannot produce the essential amino acids tyrosine and phenylalanine in the absence of mutase enzyme activity. Chorismate mutase variants permitting growth under selective conditions were expressed and purified, and their thermal denaturation profiles and catalytic efficiencies were measured. The enzyme tolerated about 34% of the possible single mutations, and the tolerance to single mutation was considerably position-dependent. When the results were compared to computational energies from design calculations, the computational procedure was found to perform quite well at selecting hydrophobic substitutions. However, known limitations of the model appear to underlie difficulties in modeling one charged residue. The availability of stability and activity data for these secondary positions helps to identify areas for improvement in computational enzyme design methodology.

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444. Engineering multispecific antibodies to recognize new forms of influenza

Barry D. Olafson, Benjamin D. Allen, Stephen L. Mayo*

The immune system can produce antibodies that bind targets with high affinity and specificity, resulting in the neutralization of infectious agents. However, an antibody can be foiled through the introduction of escape mutations in the epitope that it targets. For example, the monoclonal antibody HC19 neutralizes influenza by binding to the viral protein hemagglutinin, which is responsible for binding to the cell being infected. Several single mutations in hemagglutinin allow influenza to escape by reducing the affinity of the antibody for the epitope. The hemagglutinin mutations T131I and S157L reduce binding by about 1000-fold and 20-fold, respectively, and both mutants escape neutralization. We have been applying computational protein design methods in an attempt to generate multispecific HC19 variants that are able to bind and neutralize both the wild type and escape mutants of hemagglutinin. We are using a new multi-state design algorithm that is able to select amino acid sequences in the context of multiple structural and chemical states. For each escape position on hemagglutinin, we applied the multi-state design procedure to the antibody sequence with the requirement that it be compatible with both the wild type and the escape variant. We have also applied the same procedure to find antibody variants that might bind both a human and avian influenza hemagglutinin. The designed antibody sequences suggested by the computational procedure will be analyzed by surface plasmon resonance to determine their binding affinities for the wild type and escape mutant hemagglutinins; interesting results will be followed with biologically relevant neutralization assays. In addition to the more approximate potential functions that we use during the computational sequence selection, we have been developing a more accurate scoring procedure based on molecular dynamics simulations in explicit water. This procedure could be used to more rigorously re-rank the sequences produced by the protein design procedure. The results of the experiments described here may help to improve the predictive power of this post-processing procedure.

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445. RNA-driven association of the signal recognition particle (SRP) and SRP receptor

Alex L. Perryman, Xin Zhang, Shu-ou Shan*, Stephen L. Mayo*

Many nascent proteins contain an N-terminal signal sequence indicating that these proteins should be transported to cellular membranes for their insertion or secretion. The signal recognition particle (SRP) is a protein-RNA complex that is highly conserved in all three kingdoms of life. It binds to these signal sequences and directs the way in which the newly formed proteins are targeted. When the SRP binds to a signal sequence, elongation of the nascent polypeptide is arrested. The SRP then mediates docking of the translating ribosome with the translocation pore in the ER membrane in eukaryotes (or the plasma membrane in prokaryotes). The ribosome-nascent chain complex is directed to these membranes via its interactions with the membrane-associated SRP receptor (SR). SRP and SR both display GTPase activity, and they both act as GTPase activating proteins for each other. The SRP's RNA plays an intriguing and unprecedented catalytic role in driving the interactions between SRP and SR. It is unknown how the SRP RNA is able to accelerate this association of SRP and SR by 400-fold.

The APBS package (Adaptive Poisson-Boltzmann Solver) [1] provides numerical solutions of the Poisson-Boltzmann equation, which is one of the most widely used continuum models for characterizing the electrostatic interactions between macromolecules. APBS calculations were performed on several different crystal structures that describe aspects of the SRP-SR system. By analyzing the results of these different APBS calculations, a preliminary model was created that describes a plausible way in which RNA could accelerate the association of SRP and SR. A list of ten residues from SR and eight residues from SRP were proposed as having the potential to control this RNA catalyzed association. Xin Zhang, a graduate student in Assistant Professor Shu-ou Shan's lab, is currently performing mutagenesis and fluorescence-based experiments on these residues from SR to discern whether this model can account for the 400-fold increase in the association rates of these proteins that SRP RNA causes.

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Reference

[1] Baker, N.A., Sept, D., Joseph, S., Holst, M.J. and McCammon, J.A. (2001) *Proc. Natl. Acad. Sci. USA* **98**:10037-10041.

446. Computational design of an orthogonal androgen receptor-ligand pair

Heidi K. Privett[1], Leslie Cruz[2], Robert J. Fletterick[2], Stephen L. Mayo

Androgen receptors (AR) are nuclear hormone receptors responsible for sexual dimorphism, as well as the maintenance of skeletal and muscular systems, in response to testosterone and dihydrotestosterone (DHT). These receptors may also play a role in the development of sex-specific social and sexual behaviors in mice, but the specific neural pathways that link AR activity and behavior remain unclear [1].

In order to study the relationship between AR and sexual dimorphisms in the brains of mice that lead to these sex-specific behaviors, it would be useful to have a homologous ligand-receptor pair that is orthogonal to the wild-type ligand and receptor. Using these tools, the orthogonal AR can be introduced into the brains of mice and can be selectively activated by the addition of the

orthogonal ligand, leaving the endogenous AR systems unaffected. The Fletterick lab has synthesized a novel derivative of testosterone, which has a butyl substituant on C19 (19PT) and does not bind to the wild-type AR.

Our goal is to use the ORBIT protein design software to redesign the binding pocket of AR to accommodate binding of 19PT. All designs were carried out in the context of the 1.7 Å crystal structure of the wild-type AR bound to DHT [2]. The novel ligand was introduced into the binding pocket by overlaying it with the crystallographic position of DHT, and a library of ligand poses was generated using small rotational and translational perturbations from the initial position, as in previous work [3]. The internal flexibility of the ligand was modeled using canonical torsions for each of the rotatable bonds. We focused our design on three key positions within the binding pocket of AR that were likely to prevent 19PT from binding in the wild-type pocket. Our design of these three residues resulted in several sequences exhibiting compensatory mutations that appear to accommodate the butyl substituant of 19PT. These sequences are currently being experimentally validated by the Fletterick lab.

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References

[1] Shah, N.M., Pisapia, D.J., Maniatis, S., Mendelsohn, M.M., Nemes, A. and Axel, R. (2004) *Neuron* **43**:213-319.

[2] Hur, E., Pfaff, S.J., Payne, E.S., Gron, H., Buehrer, B.M. and Fletterick, R.J. (2004) *PLOS Biol.* **2**:1303-1312.

[3] Lassila, J.K., Privett, H.K., Allen, B.D. and Mayo, S.L. (2006) *Proc. Natl. Acad. Sci. USA* **103**:16710-16715.

447. Improved continuum electrostatics and solvation for protein design

Christina Vizcarra[*]*, Stephen L. Mayo*

Our goal is to develop accurate electrostatic models that can be implemented in current computational protein design protocols. To this end, we improve upon a previously reported pairwise decomposable, finite difference Poisson-Boltzmann (FDPB) model for protein design [1]. The improvement involves placing generic sidechains at positions with unknown amino acid identity and explicitly capturing two-body perturbations to the dielectric environment. We compare the original and improved FDPB methods to standard FDPB calculations in which the dielectric environment is completely determined by protein atoms. The generic sidechain approach yields a two- to three-fold increase in accuracy per residue or residue pair over the original pairwise FDPB implementation, with no additional computational cost. Distance dependent dielectric and solvent-exclusion models were also compared to standard FDPB energies. The accuracy of the new pairwise FDPB method is shown to be superior to these models, even after re-parameterization of the solvent-exclusion model.

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Reference

[1] Marshall S.A., Vizcarra C.L. and Mayo, S.L. (2005) *Protein Sci.* **14**:1293-1304.

448. Establishing the entatic in folding metallated azurin

Corey J. Wilson, Chenghang Zong[*]*, Peter G. Wolynes*[*]*, Stephen L. Mayo*

Understanding how the folding of proteins establishes their functional characteristics at the molecular level challenges both theorists and experimentalists. The simplest testbeds for confronting this issue are provided by electron transfer proteins. The environment provided by the folded protein to the cofactor tunes the metal's electron transport capabilities as envisioned in the entatic hypothesis. According to the entatic hypothesis, the so-called "entatic state" occurs in proteins when a group, metal or nonmetal, is forced into an unusual, energetically strained geometric or electronic state (or rack-induced state). The rigidity of the protein scaffold speeds charge transfer by minimizing the nuclear reorganization energy. To see how the entatic state is achieved, one must study how the folding landscape affects and in turn is affected by the metal. In this study, we have developed a coarse-grained Hamiltonian (i.e., a minimalist metalloprotein representation) to explicitly model how the coordination of the metal modifies the folding of *Pseudomonas aeruginosa* azurin, a blue copper protein [1]. Our free energy functional-based approach directly yields the proper non-linear extra-thermodynamic free energy relationships for the kinetics of folding the wild type as well as several point-mutated variants of the metallated protein. The results agree quite well with corresponding laboratory experiments. Moreover, our modified free energy functional provides a sufficient level of detail to explicitly model how the geometric entatic state of the metal modifies the dynamic folding nucleus of azurin.

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Reference

[1] Zong, C., Wilson, C.J., Shen, T., Wittung-Stafshede, P., Mayo, S.L. and Wolynes, P.G. (2007) *Proc. Natl. Acad. Sci. USA* **104**:3159-3164.

Publications

Allen, B.D. and Mayo, S.L. (2006) Dramatic performance enhancements for the FASTER optimization algorithm. *J. Comput. Chem*. **27**:1071-1075.

Alvizo, O., Allen, B.D. and Mayo, S.L. (2007) Computational protein design promises to revolutionize protein engineering. *Biotechniques* **42**:31-35.

Alvizo, O. and Mayo, S.L. (2007) Evaluating and optimizing computational protein design force fields using fixed composition based negative design. *Proc. Natl. Acad. Sci. USA.* In preparation.

Choi, E.J., Mao, J. and Mayo, S.L. (2007) Computational design and biochemical characterization of maize nonspecific lipid transfer protein variants for biosensor applications. *Protein Sci.* **16:**582-588.

Choi, E.J. and Mayo, S.L. (2006) Generation and analysis of proline mutants in protein G. *Protein Eng. Des. Sel.* **19**:285-289.

Hom, G.K. and Mayo, S.L. (2006) A search algorithm for fixed-composition protein design. *J. Comput. Chem.* **27**:375-378.

Huang, P., Love, J.J. and Mayo, S.L. (2007) A *de novo* designed protein-protein interface. *Protein Sci.* In press.

Lassila, J.K., Keeffe, J.R., Kast, P. and Mayo, S.L. (2007) Exhaustive mutagenesis of six secondary active-site residues in *Escherichia coli* chorismate mutase shows the importance of hydrophobic side chains and a helix N-capping position for stability and catalysis. *Biochemistry* **46**:6883-6891.

Lassila, J.K., Privett, H.K., Allen, B.D. and Mayo, S.L. (2006) Combinatorial methods for small-molecule placement in computational enzyme design. *Proc. Natl. Acad. Sci. USA* **103**:16710-16715.

Mena, M.A., Treynor, T.P., Mayo, S.L. and Daugherty, P.S. (2006) Blue fluorescent proteins with enhanced brightness and photostability from a structurally targeted library. *Nature Biotechnol.* **24**:1569-1571.

Pluckthun, A. and Mayo, S.L. (2007) The design of evolution and the evolution of design. *Curr. Opin. Struct. Biol.* **17:**1-3.

Shah, P.S., Hom, G.K., Ross, S.A., Lassila, J.K., Crowhurst, K.A. and Mayo, S.L. (2007) Full-sequence computational design and solution structure of a thermostable protein variant. *J. Mol. Biol.* **372**:1-6.

Shifman, J.M., Choi, M.H., Mihalas, S., Mayo, S.L. and Kennedy, M.B. (2006) Ca2+/calmodulin-dependent protein kinase II (CaMKII) is activated by calmodulin with two bound calciums. *Proc. Natl. Acad. Sci. USA* **103**:13968-13973.

Treynor, T.P., Vizcarra, C.L., Mena, M.A., Nedelcu, D., Olafson, B.D., Daugherty, P.S. and Mayo, S.L. (2007) Designed combinatorial libraries used to investigate mechanisms affecting electronic structure and stability of fluorescent proteins. In preparation.

Treynor, T.P., Vizcarra, C.L., Nedelcu, D. and Mayo, S.L. (2007) Computationally designed libraries of fluorescent proteins evaluated by preservation and diversity of function. *Proc. Natl. Acad. Sci. USA* **104**:48-53.

Vizcarra, C.L., Zhang, N., Marshall, S.A., Wingreen, N., Zeng, C. and Mayo, S.L. (2007) An improved pairwise decomposable finite-difference Poisson-Boltzmann method for computational protein design. *J. Comput. Chem*. Submitted.

Zollars, E.S., Marshall, S.A. and Mayo, S.L. (2006) Simple electrostatic model improves designed protein sequences. *Protein Sci*. **15**:2014-2018.

Zong, C., Wilson, C.J., Shen, T., Wittung-Stafshede, P., Mayo, S.L. and Wolynes, P.G. (2007) Establishing the entatic state in folding metallated *Pseudomonas aeruginosa* azurin. *Proc. Natl. Acad. Sci. USA* **104**:3159-3164.

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Postdoctoral Scholar: Marlene Biller
Research Laboratory Staff: Brian Blood, Ashley Grant

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Summary: We are interested in two groups of animal viruses, the alphaviruses and the flaviviruses. These viruses contain an RNA genome of about 11,000 nucleotides and are enveloped, having a lipid envelope that surrounds an icosahedral nucleocapsid. Both alphaviruses and flaviviruses are vectored by mosquitoes or other hematophagous arthropods and infect both their arthropod vectors as well as a wide range of vertebrates. We wish to understand the replication of alphaviruses and flaviviruses at a molecular level and to study the evolution of these two virus groups and more broadly the evolution of all RNA viruses.

The flaviviruses contain about 70 members, most of which are important human pathogens causing hundreds of millions of cases of human disease annually, which include hemorrhagic fevers and encephalitis. West Nile virus, which first appeared in the Americas seven years ago in New York state and then spread rapidly over most of the United States, is a member of this family. Other important flaviviruses include yellow fever virus and dengue virus. The alphaviruses consist of about 30 members of that several cause encephalitis, polyarthritis, or febrile illnesses in humans.

Although the alphaviruses and flaviviruses are not closely related, the structures of the virions in the two groups have been found recently to be related. One of the two glycoproteins present on the exterior of the virions, called E in the case of flaviviruses and E1 in alphaviruses, are structurally identical. There are other similarities in the structures of the virions in the two groups, including the formation of a heterodimer between E or E1 and the second external glycoprotein that function in an equivalent fashions. It seems clear that the virus structures, although they differ in important ways, are derived from a common ancestral source. We have suggested that this common structure may be important in the life of these viruses as arboviruses, capable of infecting both hematophagous arthropods and vertebrates.

We have started an ambitious collaboration with structural biologists at Purdue University to determine the structures of alphaviruses and flaviviruses to high resolution, and to determine the structures of all of the proteins encoded by the viruses to atomic resolution. We have now determined the structure of dengue virus to 9.5 Å and the structure of immature dengue virus that contains uncleaved prM to 11 Å. This resolution is high enough that the glycoproteins can be positioned with confidence in the virus particle, that carbohydrate side chains of the glycoproteins can be seen, and even the membrane spanning domains that anchor the glycoproteins can be identified and traced. One interesting result from such analysis is that protein E has a different bend in the virion from that in the immature virus, and that this bend also differs in the crystallized forms of E. Of even greater interest is the fact that E undergoes large structural rearrangements upon conversion of the immature virus to the mature form and again upon conversion of the mature virus to the fusion-competent form. We are extending these structural studies by constructing viruses with mutations in various proteins in order to study the interactions among proteins required for virus assembly. Such studies are important not only for our understanding of virus replication, but will be useful in the design of antivirals or of vaccines to control these important disease agents.

449. *In vitro* reactivation of immature dengue virus particles

Brian Blood, Pritsana Chomchan, Marlene Biller

During assembly of flaviviruses, the genomic RNA associates with the capsid protein to form a nucleocapsid, while the two transmembrane glycoproteins of the virus, prM and E, interact with one another in the membrane of the endoplasmic reticulum to form heterodimers. When the nucleocapsid buds into the lumen of the ER, it acquires a lipid envelope containing prM and E. These "immature" virus particles containing prM are transported to the cell surface through the Golgi apparatus. The trans-Golgi compartments are known to be mildly acid, and this acid treatment produces a conformational change in the prM protein, rendering it cleavable by furin, a host cell Golgi protease. As a final step in the maturation of the infectious particle, prM is cleaved by furin to the "pr" portion, which is lost from the particle, leaving the membrane-associated M protein of 75 amino acids. E then rearranges to form homodimers in the mature virion. It is possible to produce immature (non-infectious) virus particles that contain uncleaved prM by growing the virus in the presence of ammonium chloride. This lysosomotrophic agent causes the pH of endosomal vesicles through which virus is transported to rise, which in turn, prevents cleavage of prM by furin. In order to prove that the immature dengue particles containing prM are true intermediates in the assembly of dengue virus, we need to be able to reactivate the immature dengue virus (DEN2) *in vitro* with furin after low pH treatment. Cleavage of prM to pr and M was detected in the furin-treated particles by gel electrophoresis and silver staining, and furin treatment restored infectivity, as assayed in BHK cells by immunofluorescence. However, so far we are unable to detect reactivated particles by electron microscopy. The ultimate goal is to reactivate sufficient numbers of particles for cryo-electron reconstruction.

450. Sindbis virus E1 hinge project

Ashley Grant, Marlene Biller, Jose Corleto, Lisa Yee

Sindbis is an Alphavirus and a member of *Togaviridae* family. Sindbis is transmitted by mosquitoes, which bite and infect humans and other animals. In humans, the virus causes flu-like symptoms, such as fever and muscle ache, which quickly subside.

Sindbis virus contains a plus stranded RNA genome in an icosahedralnucleocapsid surrounded by a lipid envelope. Alphaviruses contain 240 copies of three structural proteins: two transmembranal glycoproteins (E1 and E2) and a capsid (C) protein. The C protein is complexed with the viral RNA. E1 and E2 form heterodimers on the virion surface. Each spike on the virion surface is made up of three heterodimers. E1 has three domains, I, II and III. Domain I contains the amino terminus and is located between domains II and III. The carboxy terminus lies within domain III, and the fusion peptide is at the distal end of domain II. Fusion in alphaviruses requires the dissociation of E1-E2 heterodimers and the formation of E1 homotrimers. These rearrangements require large conformational changes in the E1 protein that include changes in the hinge region between domains I and III. This hinge contains five conserved glycines. Our project is to alter the flexibility of the E1 protein in the hinge region by mutating these glycines, which are small, uncharged residues, to lysines, which are bulky charged moieties. We can then study the effects of these mutations on virus growth.

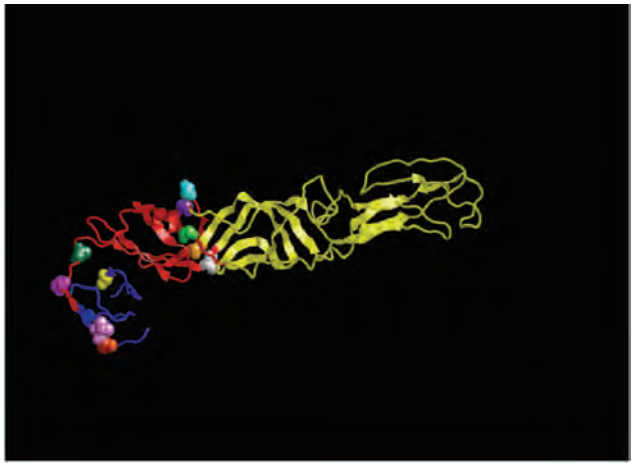


Figure 1. Crystal structure of Sindbis E1 protein with Domain I in red; Domain II in yellow; and, Domain III in blue. The different mutations are shown in the following color scheme: S0-white, S1-orange, S2-cyan, S3-purple, S4-green, S5-greenblue, S6-magenta, S7-violet, S8-redorange, and S9-yellow.

The mutations are being introduced by site-directed mutagenesis into a shuttle vector in which DNA encoding Sindbis E1 has been inserted into the PCR 2.1 vector. The mutations are: S0 (E1 aa 35 S to K), S1 (E1 aa 132 G to K), S2 (E1 aa 150 G to K), S3 (E1 aa 164 G to K), S4 (E1 aa 274 G to K), S5 (E1 aa 293 A to K), S6 (E1 aa 297 S to K), S7 (E1 aa 303 V to K), S8 (E1 aa 304 V to K), and S9 (E1 aa 326 G to K). Currently seven out of these ten mutations have been inserted into the shuttle and confirmed by DNA sequencing. The mutation will then be cloned into the full-length DNA clone of Sindbis [TE12]. After all the mutations are correctly cloned into Sindbis we will perform *in vitro* transcription to transcribe the DNA to RNA. The RNA will be transfected (Invitrogen Lipofectamine 2000) into BHK cells (Baby Hamster Kidney). After 24 to 48 hours the virus will be harvested. Immunofluorescence will be done to find out the transfection efficiency and to see if the virus particles can infect other cells. The number of infectious particles in the supernatant will be determined by plaque assay and compared with the yield from a wild-type transfection under the same conditions. If the number of particles is high enough, virions will be purified by polyethylene glycol (PEG) precipitation, followed by a sucrose gradient centrifugation. We hope to be able to produce sufficient highly purified virus particles from the mutants to examine by cryo-electron microscopy.

451. Generation of alphaviruses containing PE2 glycoproteins

Marlene Biller, Brian Blood, Ashley Grant

We are currently working with three different alphaviruses: Sindbis, Ross River, and Aura virus. They are members of the *Togaviridae* family. They contain a plus-strand RNA genome and a lipid envelope that surrounds an icosahedral nucleocapsid. Alphaviruses contains 240 copies each of three structural proteins: two transmembrane glycoproteins (E1 and E2) and a capsid protein. The structural proteins of alphaviruses are translated from a 26S subgenomic RNA as a polyprotein. The capsid protein is cleaved by the viral capsid autoprotease shortly after synthesis, and PE2 (precursor of E2) and E1 are produced in the endoplasmic reticulum, and rapidly associate to form (PE2/E1) three heterotrimers. PE2 is cleaved by furin in a post Golgi compartment to form E2 and E3 before the trimers are delivered to the plasma membrane,

The last four amino acids of E3 contain the furin recognition sequence (Arg-X-Arg-Arg or Arg-X-Lys-Arg) in almost all alphaviruses. PE2-containing particles have been produced for Semliki Forest virus and shown to be non-infectious. We want to produce PE2-containing particles of Sindbis, Ross River, and Aura for structural studies by removing the furin cleavage sites between E3 and E2 in full-length cDNA clones. This can be done in a number of ways: mutating individual basic amino acids within the site; creating an N-glycosylation site (N-X-T/S) that will interfere with cleavage; or deleting the site. We have Sindbis mutants generated in all of these ways, and have Ross River mutants with the new N-glycosylation site and the deletion.

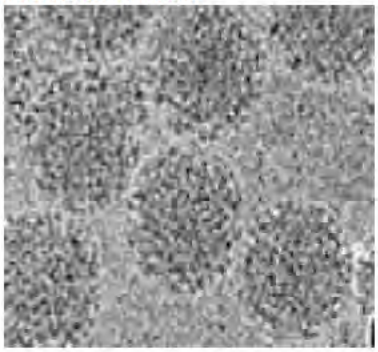
We have cloned all the Sindbis and Ross River mutants into full-length cDNA constructs. The Sindbis mutants produced infectious virus, but grew to a lower titer

than wt after transfection. We have examined the virions by protein gel electrophoresis and Western blot with anti E2 antibody, and showed that they contained PE2. We have sent preparations of the purified Sindbis deletion mutant and the Sindbis N-glycosylation mutant to Purdue University for EM and Cryo EM. The first EM pictures showed that the Sindbis deletion mutant lacked the "cross-hatch" pattern characteristic of wt, and a cryo-electron reconstruction of the PE2-containing particles from the deletion mutant was obtained.

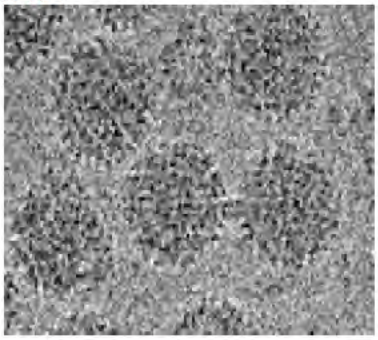
We are very interested in the N-glycosylation mutants, because the additional glycan might be visible on the Cryo-EM, and thereby showing its position on the particle.

The Ross River deletion mutant was not infectious. We have collected and purified particles produced after electroporation. The Ross River N-glycosylation mutant was less infectious that Wt. So far we have not been able to produce sufficient PE2-containing Ross River particles for a reconstruction.

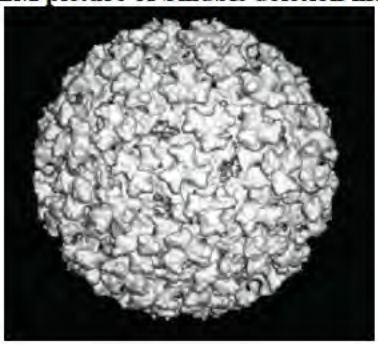
Sindbis deletion mutant



Sindbis WT



Cyro EM picture of Sindbis deletion mutant



452. Flexibility of yellow fever E protein

Ashley Grant, Marlene Biller

The flavivirus E protein adapts different conformations in the immature state, in mature virus particles and in its low pH "fusion competent" configuration. Immature flavivirus particles contain 60 trimers of prM-E heterodimers. The prM protein covers the E protein, and protects it from undergoing premature fusion when passing through the acidic compartments and vesicles of the trans Golgi network. Following cleavage of prM by furin the virus enters its mature state in which the E proteins form 90 head-to-tail dimers that lie parallel to and completely cover the viral membrane. After entering cells by receptor-mediated endocytosis, fusion with the host membrane is initiated by low pH-induced conformational changes in the E protein. Thus, flexibility of the E protein is a functional requirement for both assembly and infection. Zhang *et al.* (*Structure*, Vol.12, September, 2004) compared crystal structures of dengue sE and the structure of E monomers in the immature and mature virions and found that the E protein can bend about a hinge between domains II and domain I+III. Their study showed that during maturation, domain DI+DIII reorients with respect to the viral membrane, and that this movement is accompanied by a change of 27°in the angle between DII and DI+DIII. Moreover, between E protein in mature virus and E protein in the fusion competent state the hinge angle changes by 37°.

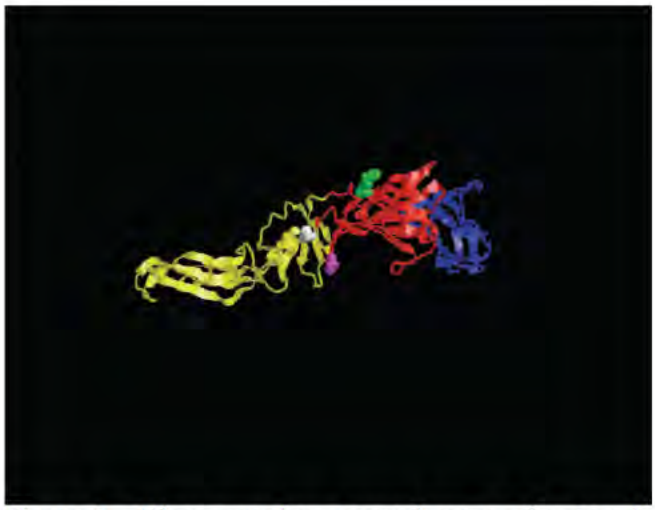


Figure 1. Hinge mutations mapped out on the crystal structure of the E protein of TBE. The H1 mutation site shown in white; H3 shown in magenta; and, H4 shown in green. The colors of the chains are different with each region. Domain I is red, Domain II is yellow and Domain III is blue.

Zhang *et al.* noted the presence of conserved glycines in the hinge region, between domain I and domain II. Since the flexibility of the E protein of flaviviruses is so important for the virus in maturation and fusion, we believe that these small, uncharged glycines and alanines can be of great significance for the E protein. We have mutated these glycines and alanines to amino acids that we

think could affect flexibility of the E protein but perhaps not destroy its overall conformation. The different hinge mutants are: H1-1 (E aa54 A to I), H1-2 (E aa54 A to K), H3-1 (E aa 187 A to I), H3-2 (E aa 187 A to K), H4-1 (E aa 279 G to I), H4-2 (E aa 279 G to K), H4-3 (E aa 279 G to T).

All mutants are now in the full-length yellow fever vector and have been sequenced. We have transcribed RNA from the mutants in full-length virus, and transfected BHK cells. RNA replication was detected by immunofluorescence, and virus infectivity checked by plaque assay. The H11, H12, H31, H41 and H43 mutants were infectious, but with a reduced titer relative to wild type. On the other hand, H32 and H42 do not produce infectious particles, implying that the single amino acid change that we introduced has had big impact on the infectivity, which could mean that flexibility of the E protein is affected. We need to determine whether any non-infectious viral particles are produced, and if so we plan to prepare sufficient quantities of them for cryo-electron microscopy.

453. The function of yellow fever envelope protein in flavivirus assembly

Marlene Biller

The family *Flaviviridae* contains many important human pathogens including yellow fever, West Nile, dengue, and tick-borne encephalitis viruses. Flavivirus are enveloped positive-stranded RNA viruses. The viral envelope (E) protein of yellow fever virus has several major functions, including receptor attachment and membrane fusion, and also carries the major antigenic epitopes leading to a protective immune response. E proteins in mature virions form 90 head-to-tail dimers that lie parallel and completely cover the viral membrane. Dimers of E protein have also been fitted into the cryo-electron density envelope of dengue virus and there has been interest in determining how important dimer formation by E protein is for the overall configuration (and perhaps the stability) of flaviviruses. The E protein has an elongated shape and is composed of three major parts: a central domain that contains the N-terminal region (domain I); the fusion and dimerization domain (domain II); and a C-terminal putative receptor-binding domain (domain III). In mature virions, the fusion peptide is probably protected by the Domain III of the opposite E protein in the dimer. A set of mutants has been made to investigate dimer formation. These mutants are in contact regions of the homodimer and were carefully selected, based on where they are located in the protein and whether they are conserved among flaviviruses. We have nine mutations to the current time. Three are in domain II: F1 Mutation at E aa251 E to K; F2 Mutation at E aa251 E to Q; and F3 mutation at E aa253 S to P to insert a helix breaker. There are four in domain III: F4 Deletion aa 313-314; F4a mutation aa 313-314 to two Ala; F5 Deletion aa. 313-316; F7 Mutation at E aa311 T to A, and, F8 mutation at E aa319 V to D. F6 consists of an insertion of three Ala residues at aa 155 in domain I. The mutations were made in a transient expression vector, and all of them have been introduced into the full-length yellow fever virus clone (pACNR vector). RNA is transcribed *in vitro* and used to transfect BHK cells. RNA replication is detected by immunofluorescence, and virus infectivity is checked by plaque assay.

All the mutants in domain III altered the infectivity. F4 and F5 were not infectious. Immunofluorescence of the F5 mutant show that the E protein is trapped somewhere in the cell, and are probably not released to the surface. A mutant called F4a was made, where aa T313-G314 were replaced by alanines, instead of deleted, as in F4. F4a had very low infectivity. Immunofluorescence showed primarily single cells were transfected, so the infectivity is probably caused by revertants. RT-PCR will be done to check the sequence of virus in the revertant plaques. F7 and F8 also had low infectivity. The infectivity of F8 was probably caused by revertants. The domain III area is very important for the virus, and the areas we have been looking at probably cover the fusion peptide in the opposite E protein. Mutant F1 in domain II had nearly no infectivity when titered with plaque assay of 1h transfected cells, but infectivity increased in cells transfected for 3 days, probably due to revertants. Mutants F2 and F3 in domain II had low infectivity. Mutant F6 in domain I was less infectious than wild type and gave very small plaques relative to wild type. Next we will purify the virus and crosslink the E proteins to see if they produce dimers. We have also started combining certain mutations into double mutants, such as putting F4a and F1 together in one construct. Although the fact that revertants arise so easily is a complication for some types of experiments, it will be of interest to sequence the revertants, and map any second-site compensating mutations that have arisen, and determine their effect on the ability of the E protein to dimerize.

454. *In vitro* reconstitution of an enveloped animal virus

Odisse Azizgolshani[*], William M. Gelbart[*], Charles M Knobler[*], Marlene Biller

The goal of this project is to assemble an infectious, enveloped virus *in vitro* from purified components. We have been attempting to do so by wrapping a viral membrane (VM) around the nucleocapsid (NC), thereby mimicking the process of viral budding, the event that completes the life cycle of many animal viruses and that results in the release of mature, infectious virions. We hypothesize that budding *in vivo* is driven by the interaction of capsid proteins - assembled into NCs in the cytoplasm of infected cells - with the spike proteins in the plasma membrane; specifically, we hypothesize that no active processes or host factors are involved. We have chosen Sindbis virus (SINV) for this study due to its structural simplicity and the fact that SINV NC has been assembled *in vitro*. SINV NC is made of a ssRNA genome and 240 identical copies of one capsid protein. The VM is a phospholipid bilayer derived from the host cell, in which

240 copies of two virally encoded glycoproteins (E1 and E2) are inserted as heterodimers (which assemble into trimers in the VM). *In vitro* assembly could have important medical applications, in that both the VM could be tailored to target specific tissues and the NCs could be independently modified to contain therapeutic genes.

Our initial approach was to separate the VM from the NC by treating the intact virions with detergent, followed by detergent removal to allow the rewrapping event. We had assumed that the glycoproteins would stay in the VM during detergent treatment - i.e., that the VM would remain intact. Although the detergents used (Triton X-100 and octyl glucoside) are capable of disrupting the NC-spike interactions, we could not show that the VM was intact. As an alternative approach, we have started reconstituting artificial membranes with glycoproteins to form virosomes. It has been shown that the SINV glycoproteins are inserted into lipid vesicles vectorially, with their glycosylated ends facing outward - as in the VMs - and that the glycoproteins retain their native function, namely the ability to bind to host cells and to facilitate membrane fusion when triggered by acidic pH. We are currently attempting to make vesicles containing NCs, such that each vesicle contains a single NC on average. The glycoproteins will then be added and assembly of infectious particles from these non-infectious components (NC, purified glycoproteins, lipid vesicle) will be determined by plaque assays as well as by structural studies (EM). In a parallel effort, "added-length mutants" of Sindbis have been constructed, in which arbitrary numbers of nucleotides have been inserted into the 3' noncoding region, just downstream of the open reading frame for glycoprotein E1. These will be used to measure the efficiency of NC reconstitution *in vitro* (genome packaging) as a function of added length.

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Publication

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Summary: Our main subject is the ubiquitin system. The field of ubiquitin and regulated protein degradation was created in the 1980s, largely through the complementary discoveries by the laboratory of A. Hershko (Technion, Haifa, Israel) and by my laboratory, then at MIT. These discoveries revealed three sets of previously unknown facts:

1. That ATP-dependent protein degradation involves a new protein modification, ubiquitin conjugation, which is mediated by specific enzymes, termed E1, E2 and E3.
2. That the selectivity of ubiquitin conjugation is determined by specific degradation signals (degrons) in short-lived proteins, including the degrons that give rise to the N-end rule.
3. That ubiquitin-dependent processes play a strikingly broad, previously unsuspected part in cellular physiology, primarily by controlling the *in vivo* levels of specific proteins. Ubiquitin conjugation was demonstrated by us to be required for the protein degradation *in vivo*, for cell viability, and also, specifically, for the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses. We also cloned and analyzed the first ubiquitin genes, the first specific E3 ubiquitin ligase (UBR1), the first deubiquitylating enzymes (UBP1 and UBP2), and identified the first physiological substrate of the ubiquitin system, the MATalpha2 transcriptional repressor. In addition, we showed that ubiquitin-dependent proteolysis involves a substrate-linked polyubiquitin chain of unique topology that is required for protein degradation. The ubiquitin system was also discovered to possess *subunit selectivity*, i.e., the ability to destroy a specific subunit of a multisubunit protein, leaving the rest of the protein intact and thereby making possible *protein remodeling*. This fundamental process underlies the cell cycle (the replacement of cyclin subunits in cell-cycle kinases), the activation of transcription factors such as, for example, NF-kappaB, and a multitude of other biological pathways.

The Hershko laboratory produced the first of these fundamental advances (item 1), and my laboratory produced the other two (items 2 and 3). Over the last ~15 years, these complementary discoveries led to the enormous expansion of the ubiquitin field, which became one of the largest arenas in biomedical science, the point of convergence of many disparate disciplines. (For accounts of the early history of the ubiquitin field, see Hershko *et al*., 2000; Varshavsky, 2006). Our biological discoveries of the 1980s, together with later studies by many excellent laboratories that entered the field in the 1990s and afterward, yielded the modern paradigm of the central importance of regulated proteolysis for the control of the levels of specific proteins *in vivo*, as distinguished from their control by transcription and protein synthesis. In other words, these advances revealed that the control through regulated protein degradation rivals, and often surpasses in significance the classical regulation through transcription and translation. This radically changed understanding of the design of biological circuits is beginning to have a major impact on medicine, given the astounding functional range of the ubiquitin system and the multitude of ways in which ubiquitin-dependent processes can malfunction in disease or in the course of aging, from cancer and neurodegenerative syndromes to perturbations of immunity and many other illnesses, including birth defects. Our work at Caltech continues to focus on ubiquitin-dependent processes.

The effect of an intracellular protein on the rest of a cell depends on the protein's concentration. The latter is determined by the rate of synthesis and/or import of the protein in relation to the rates of its degradation, inactivation by other means, or export from the compartment. The *in vivo* half-lives of intracellular proteins range from a few seconds to many days. Over the last decade, a vast number of biological circuits were shown to contain either constitutively or conditionally short-lived proteins. In addition, damaged or otherwise abnormal intracellular proteins tend to be recognized as such and selectively degraded, largely by the same ubiquitin-proteasome system that mediates the proteolysis of (conditionally) short-lived regulatory proteins. The metabolic instability of a regulator provides a way to generate its spatial gradients and allows for rapid adjustments of its concentration (or subunit composition) through changes in the rate of its degradation.

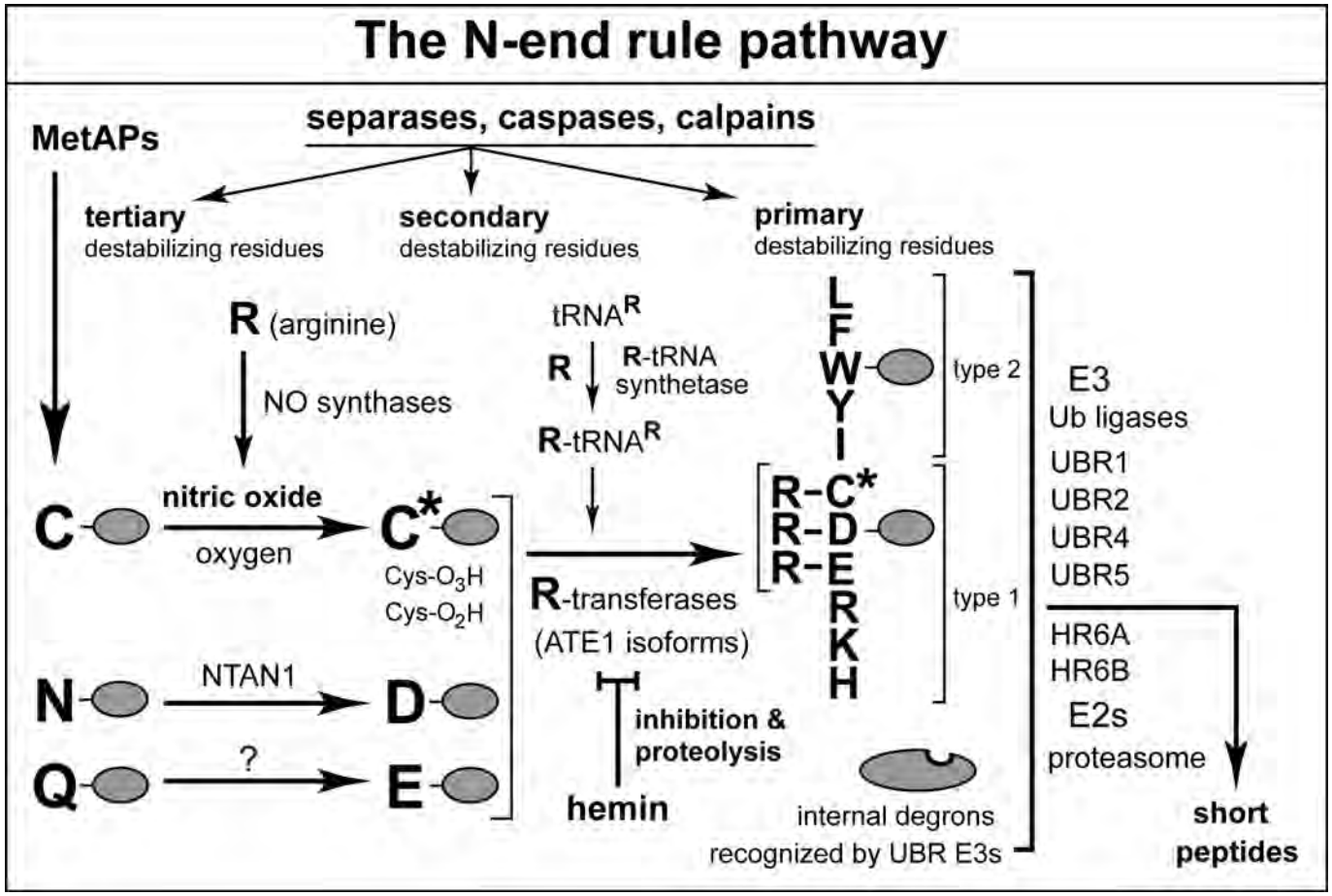

Figure 1. The N-end rule pathway in mammals. N-terminal residues are indicated by single-letter abbreviations for amino acids. The ovals denote the rest of a protein substrate. MetAPs, methionine aminopeptidases. A notation, upward of *"hemin"* in the middle of diagram, is a modified "down-regulation" sign, to denote down-regulation that is mediated, at least in part, by target's degradation (Hu *et al.*, 2007). MetAPs, Met-aminopeptidases. C* denotes oxidized Cys, either Cys-sulfinate or Cys-sulfonate, produced in reactions mediated by nitric oxide (NO), oxygen (O_2) and their derivatives (Hu *et al.*, 2005). Type 1 and type 2 primary destabilizing N-terminal residues are recognized by multiple E3 Ub ligases of the N-end rule pathway, including UBR1 and UBR2 (Tasaki *et al.*, 2005). Through their other substrate-binding sites, these E3s also recognize internal (non-N-terminal) degrons in other substrates of the N-end rule pathway, denoted by a larger oval.

Ubiquitin (Ub) is a 76-residue protein that exists in cells either free or conjugated to many other proteins. Degradation of intracellular proteins by the Ub-proteasome system regulates a multitude of processes: cell growth; cell division and differentiation; signal transduction; responses to stress; and a broad range of metacellular (organismal) processes, such as embryonic development, immunity and the functions of the nervous system. Ub-dependent proteolysis involves the "marking" of a substrate through covalent conjugation of Ub to a substrate's internal Lys residue. Ub conjugation is mediated by the E1-E2-E3 enzymatic cascade. E1, the ATP-dependent Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a specific Cys residue of E1. In the second step, activated Ub is transesterified to a Cys residue of a Ub-conjugating (E2) enzyme. Thereafter a complex of E2 and another enzyme, E3 (this complex, or E3 alone, is called a Ub ligase), conjugates Ub to a Lys residue of a substrate. The numerous proteolytic pathways of the Ub system have in common their dependence on Ub conjugation and the 26S proteasome (which processively degrades Ub-protein conjugates), and differ largely through their utilization of distinct E2-E3 complexes. Specific E3s recognize (bind to) specific degradation signals (degrons) of their substrates. The diversity of E3s and E2s (there are more than a thousand of distinct E3s in a mammal) underlies the enormous range of substrates that are recognized and destroyed by the Ub system, in ways that are regulated both temporally and spatially. Ub has nonproteolytic functions as well.

One pathway of the Ub system is the N-end rule pathway (Fig. 1). This pathway recognizes a range of degrons, including a set called N-degrons. In eukaryotes, an N-degron consists of three determinants: a destabilizing N-terminal residue of a protein substrate; its internal Lys (K) residue(s) (the site of formation of a poly-Ub chain); and a conformationally flexible region(s) in the vicinity of these determinants that is required for the substrate's ubiquitylation and/or degradation. The set of

destabilizing residues in a given cell type yields a rule, called the N-end rule, which relates the *in vivo* half-life of a protein to the identity of its N-terminal residue.

The N-end rule has a hierarchic structure (Fig. 1). In eukaryotes, N-terminal Asn (N) and Gln (Q) are tertiary destabilizing residues (denoted as Nd^t) in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp (D) and Glu (E) (denoted as Nd^s). Destabilizing activity of N-terminal Asp and Glu requires their conjugation, by *ATE1*-encoded isoforms of Arg-tRNA-protein transferase (arginyl-transferase or R-transferase), to Arg (R), one of the primary destabilizing residues (denoted as Nd^p). The latter N-terminal residues are recognized by E3 Ub ligases of the N-end rule pathway, called N-recognins. The N-end rule pathway of the yeast *Saccharomyces cerevisiae* is mediated by a single N-recognin, UBR1, while at least four N-recognins, including UBR1, mediate this pathway in mammals (Fig. 1) (Tasaki *et al*., 2005).

An N-degron is produced from pre-N-degron through a proteolytic cleavage. Methionine aminopeptidases (MetAPs) remove Met from the N-terminus of a newly formed protein if the residue at position 2, to be made N-terminal after cleavage, has a small enough side chain. Consequently, amongst the 13 destabilizing residues of the mammalian N-end rule (Fig. 1), only Cys can be made N-terminal by MetAPs. (Any destabilizing residue, including Cys, can be made N-terminal through internal cleavages of proteins by other proteases, such as separases, caspases, and calpains (Fig. 1).) Our previous work has shown that in mammals and other eukaryotes that produce nitric oxide (NO), the set of arginylated residues contains not only Asp and Glu, but also N-terminal Cys (C), which is arginylated after its oxidation (Kwon *et al*., 2002). The *in vivo* oxidation of N-terminal Cys requires NO, as well as oxygen (O_2) or its derivatives (Fig. 1) (Hu *et al*., 2005).

Although prokaryotes lack Ub conjugation and Ub itself, they contain (Ub-independent) N-end rule pathways. The sets of destabilizing residues in prokaryotic N-end rules contain secondary destabilizing (Nd^s) residues as well. Their identities (Arg and Lys in *E. coli*; Arg, Lys, Asp and Glu in some other prokaryotes) are either overlapping with, or distinct from, the eukaryotic Nd^s residues (Graciet *et al*., 2006). The activity of Nd^s residues in prokaryotes requires their conjugation to Leu (an Nd^p residue) by Leu-tRNA-protein transferases (L-transferases), in contrast to the conjugation of Arg to N-terminal Asp, Glu, or (oxidized) Cys in eukaryotes (Fig. 1). Prokaryotic L-transferases are of two distinct kinds, differing in both amino acid sequence and substrate specificity. Remarkably, L-transferases of one class, encoded by *bpt* genes, are sequelogs of *ATE1*-encoded eukaryotic R-transferases, despite the fact that Bpt (prokaryotic) aminoacyl-transferases conjugate Leu, rather than Arg, to the N-termini of cognate substrates (Graciet *et al*., 2006).

In the above terminology (Varshavsky, 2004), "sequelog" and "spalog" denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a 3D structure that is similar, to a specified extent, to another 3D structure. "Sequelog" and "spalog" are mnemonically helpful, single-word terms whose rigor-conferring advantage is their *evolutionary and functional neutrality*. The sequelog terminology conveys the fact of sequence similarity (sequelogy) without evolutionary or functional connotations, in contrast to interpretation-laden terms such as "homolog," "ortholog," and "paralog." The latter terms are compatible with the sequelog/spalog terminology and can be employed to convey understanding about functions and common descent, if this information is available (Varshavsky, 2004).

The functions of the N-end rule pathway include the regulation of import of short peptides (through the degradation, modulated by peptides, of the import's repressor), the fidelity of chromosome segregation (through the degradation of a conditionally produced cohesin fragment), the regulation of apoptosis (through the degradation of a caspase-processed inhibitor of apoptosis), the regulation of meiosis, the leaf senescence in plants, as well as neurogenesis and cardiovascular development in mammals. M. Zenker and colleagues have discovered, in a collaboration that included our lab as well, that mutations in human UBR1, one of several functionally overlapping N-recognins (Fig. 1), are the cause of Johanson-Blizzard Syndrome (JBS), which comprises mental retardation, physical malformations, and severe pancreatitis (Zenker *et al*., 2005). Abnormalities of the previously constructed *UBR1*$^{-/-}$ mouse strains (Kwon *et al*., 2001) include pancreatic insufficiency, a less severe version of the defect in human JBS (*UBR1*$^{-/-}$) patients (Zenker *et al*., 2005). The cardiovascular and other functions of the N-end rule pathway involve the arginylation-mediated degradation of RGS4, RGS5, and RGS16 (Hu *et al*., 2005; Lee *et al*., 2005). These "GTPase-activating" proteins function by inhibiting the signaling by specific G proteins, and are themselves down-regulated through the NO/O_2-dependent degradation by the N-end rule pathway (Hu *et al*., 2005). The N-terminal Cys residues of RGS4, RGS5 and RGS16 are oxidized *in vivo* at rates controlled by NO and oxygen, followed by the arginylation of oxidized Cys and processive proteolysis by the rest of the N-end rule pathway (Fig. 1). This pathway is also essential for chromosome stability, through its targeting and selective destruction of a separase-produced C-terminal fragment of a cohesin's subunit that bears a destabilizing N-terminal residue (Rao *et al*., 2001). While the identity of the key residue that becomes N-terminal upon cleavage by separase varies amongst cohesins of different eukaryotes, it is a destabilizing residue in all eukaryotes examined, indicating the functional importance of maintaining a short *in vivo* half-life of the cohesin fragment. In particular, the N-end rule pathway is also required for degradation of the *in vivo*-produced fragment of the mammalian RAD21/SCC1 subunit of cohesin (J. Zhou, J. Sheng, R.-G. Hu and A. Varshavsky, unpublished data). Most recently, the arginylation branch of the N-end rule pathway was discovered to be, in addition, a sensor of heme (Hu *et al*., 2007) (see Hu abstract).

Functional and mechanistic studies of the N-end rule pathway in yeast and mammals are a major theme of our current work.

References

Graciet, E. Hu, R.-G., Piatkov, K., Rhee, J.H., Schwarz, E.M. and Varshavsky, A. (2006) *Proc. Natl. Acad. Sci. USA* **103**:3078-3083.

Hershko, A., Ciechanover, A. and Varshavsky, A. (2000) *Nature Med*. **6**:1073-1081.

Hu, R.G., Sheng, J., Qi, X., Xu, Z., Takahashi, T.T. and Varshavsky, A. (2005) *Nature* **437**:981-986.

Hu, R.G., Wang, H. and Varshavsky, A. (2007). Submitted for publication.

Kwon, Y.T., Kashina, A.S. and Varshavsky, A. (1999) *Mol. Cell. Biol*. **19**:182-193.

Kwon, Y.T., Xia, Z., Davydov, I.V., Lecker, S.H. and Varshavsky, A. (2001) *Mol. Cell. Biol.* **21**:8007-8021.

Rao, H., Uhlmann, F., Nasmyth, K. and Varshavsky, A. (2001) *Nature* **410**:955-959.

Tasaki, T., Mulder, L.C.F., Iwamatsu, A., Lee, M.J., Davydov, I.V., Varshavsky, A., Muesing, M. and Kwon, Y.T. (2005) *Mol. Cell. Biol.* **25**:7120-7136.

Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999) *Nature* **400**:37-42.

Varshavsky, A. (2004) *Curr. Biol*. **14**:R181-R183.

Varshavsky, A. (2006) *Protein Sci.* **15**:647-654.

455. Arginyl-transferase: Specificity, putative substrates, bidirectional promoter, and splicing-derived isoforms

Rong-Gui Hu, Christopher Brower, Haiqing Wang, Ilia V. Davydov[1], Jun Sheng, Jianmin Zhou, Yong Tae Kwon[2], Alexander Varshavsky

Substrates of the N-end rule pathway include proteins with destabilizing N-terminal residues. Three of them – Asp, Glu and (oxidized) Cys – function through their conjugation to Arg, one of destabilizing N-terminal residues that are recognized directly by the pathway's ubiquitin ligases. The conjugation of Arg is mediated by arginyl-transferase, encoded by *ATE1*. Through its regulated degradation of specific proteins, the arginylation branch of the N-end rule pathway mediates, in particular, the cardiovascular development, the fidelity of chromosome segregation, and the control of signaling by nitric oxide (see Introduction). We found that mouse *ATE1* specifies at least six mRNA isoforms, which are produced through alternative splicing, encode enzymatically active arginyl-transferases, and are expressed at varying levels in mouse tissues. We also found that the *ATE1* promoter is bidirectional, mediating the expression of both *ATE1* and an oppositely oriented, previously uncharacterized gene. In addition, we identified glucose-regulated protein 78 (GRP78) and protein disulfide isomerase (PDI) as putative physiological substrates of arginyl-transferase. Purified isoforms of arginyl-transferase that contain the alternative first exons differentially arginylate these proteins in extract from *ATE1*$^{-/-}$ embryos, suggesting that specific isoforms may have distinct functions. While the N-end rule pathway is confined to the cytosol and the nucleus, and although GRP78 and PDI are located largely in the endoplasmic reticulum, recent evidence suggests that these proteins are also present in the cytosol and other compartments *in vivo*, where they may become N-end rule substrates.

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References

Hu, R.G., Sheng, J., Qi, X., Xu, Z., Takahashi, T.T. and Varshavsky, A. (2005) *Nature* **437**:981-986.

Hu, R.-G., Brower, C.S., Wang, H., Davydov, I.V, Sheng, J., Zhou, J., Kwon, Y.T. and Varshavsky, A. (2006) *J. Biol. Chem.* **281**:32559-32573.

456. Mechanistic and functional studies of N-terminal arginylation

Christopher Brower, Jianmin Zhou, Rong-Gui Hu, Haiqing Wang, Jun Sheng, Alexander Varshavsky

This Abstract mentions some of our ongoing projects that aim to further advance the understanding of the arginylation branch of the N-end rule pathway, beyond the understanding described above.

(**i**) Construction and functional analyses of mouse strains (and cells derived from them) in which the expression of *ATE1*-encoded Arg-tRNA-protein transferases (R-transferases) is selectively and conditionally abolished in specific cell lineages during embryogenesis, or postnatally (*Christopher Brower*). This set of projects should make possible, among other things, a functional dissection of N-terminal arginylation in specific organ systems and cell types of adult mice. (A nonconditional *ATE*$^{-/-}$ genotype is embryonic lethal (Kwon *et al.*, 2002).

(**ii**) Construction and functional analysis of knock-in mouse strains that contain a doxycycline-inducible allele of *ATE1*, and thus, can overproduce R-transferases, in a controllable manner, in specific cell types during embryogenesis, or postnatally (*Jianmin Zhou, Christopher Brower*).

(iii) Analysis of chromosome stability and regulation of apoptosis in mouse *ATE1*$^{-/-}$ cells (*Jianmin Zhou, Jun Sheng, Cory Hu, and Christopher Brower*). These projects stem from the discovery of the function of the *S. cerevisiae* N-end rule pathway in the maintenance of chromosome stability (Rao *et al*., 2001), and from the conjecture that an analogous function in mammalian cells involves the (*ATE1*-dependent) arginylation branch of the N-end rule pathway. Recent work indicated that N-terminal arginylation is essential for the *in vivo* degradation of the separase-produced fragment of SCC1/RAD21, a subunit of mouse cohesin. Moreover, *ATE1(-/-)* fibroblasts were also found exhibit a strong chromosome instability (CIN), presumably because, at least in part, of their inability to destroy the separase-produced cohesin's fragment (*Jianmin Zhou*).

(iv) Identification of *ATE1*-dependent circuits (i.e., circuits that involve N-terminal arginylation) through

the identification of mouse genes whose expression is significantly altered during embryonic development in *ATE-/-* embryos, using microarray techniques, differential display and analogous methods with *ATE(-/-*) and congenic +/+ embryos or EF cells (*Cory Hu, Jun Sheng, Jianmin Zhou*).

References

Hu, R.G., Sheng, J., Qi, X., Xu, Z., Takahashi, T.T. and Varshavsky, A. (2005) *Nature* **437**:981-986.

Hu, R.-G., Brower, C.S., Wang, H., Davydov, I.V, Sheng, J., Zhou, J., Kwon, Y.T. and Varshavsky, A. (2006) *J. Biol. Chem*. **281**:32559-32573.

Kwon, Y.T., Kashina, A.S., Davydov, I.V., Hu, R.-G., An, J.Y., Seo, J.W., Du, F. and Varshavsky, A. (2002) *Science* **297**:96-99.

Rao, H., Uhlmann, F., Nasmyth, K. and Varshavsky, A. (2001) *Nature* **410**:955-959.

457. New aminoacyl-transferases and the N-end rule pathway of prokaryotic/eukaryotic specificity in a human pathogen

Emmanuelle Graciet[1], Rong-Gui Hu, Konstantin Piatkov, Joon Haeng Rhee[2], Erich Schwarz[3], Alexander Varshavsky

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue (see Introduction). Primary destabilizing N-terminal residues (Nd^p) are recognized directly by the targeting machinery. The recognition of secondary destabilizing N-terminal residues (Nd^s) is preceded by conjugation of an Nd^p residue to Nd^s of a polypeptide substrate. In eukaryotes, *ATE1*-encoded arginyl-transferases ($R^{D,E,C*}$-transferases) conjugate Arg (R), an Nd^p residue, to Nd^s residues Asp (D), Glu (E) or oxidized Cys (C*) (Fig. 1). Ub ligases recognize the N-terminal Arg of a substrate and target the (ubiquitylated) substrate to the proteasome. In prokaryotes such as *Escherichia coli*, Nd^p residues Leu (L) or Phe (F) are conjugated, by the *aat*-encoded Leu/Phe-transferase ($L/F^{K,R}$-transferase), to N-terminal Arg or Lys, which are Nd^s in prokaryotes but Nd^p in eukaryotes. In prokaryotes, substrates bearing the Nd^p residues Leu, Phe, Trp or Tyr are degraded by the proteasome-like ClpAP protease (Tobias *et al*., 1991; Shrader *et al*., 1993).

Despite enzymological similarities between eukaryotic $R^{D,E,C*}$-transferases and prokaryotic $L/F^{K,R}$-transferases, there is no significant sequelogy (sequence similarity) between them (see a brief description, in square parentheses in Introduction, of the recently introduced terms "sequelog" and "spalog" (Varshavsky, 2004). We identified a new aminoacyl-transferase, termed Bpt, in the human pathogen *Vibrio vulnificus* (Graciet *et al*., 2006). Although it is a sequelog of eukaryotic $R^{D,E,C*}$-transferases, this prokaryotic transferase was found to exhibit a "hybrid" specificity, conjugating Nd^p Leu to Nd^s Asp or Glu, the first such Nd^s residues in prokaryotes. Another new aminoacyl-transferase, termed ATEL1, of the eukaryotic pathogen *Plasmodium falciparum*, is a sequelog of prokaryotic $L/F^{K,R}$-transferases (Aat), but has the specificity of eukaryotic $R^{D,E,C*}$-transferases (ATE1) (Graciet *et al*., 2006). In addition, our phylogenetic analysis suggests that the substrate specificity of R-transferases arose by two distinct routes during the evolution of eukaryotes (Graciet *et al*., 2006).

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References

Graciet, E. Hu, R.-G., Piatkov, K., Rhee, J.H., Schwarz, E.M. and Varshavsky, A. (2006) *Proc. Natl. Acad. Sci. USA* **103**:3078-3083.

Shrader, T.E., Tobias, J.W. and Varshavsky, A. (1993) *J. Bact.* **175**:4364-4374.

Tobias, J.W., Shrader, T.E., Rocap, G. and Varshavsky, A. (1991) *Science* **254**:1374-1377.

Varshavsky, A. (2004) *Curr. Biol.* **14**:R181-R183.

458. Biochemical and genetic studies of UBR3, a ubiquitin ligase with a function in olfactory and other sensory systems

Takafumi Tasaki[*], Yong Tae Kwon[*], Alexander Varshavsky

Our previous work identified E3 ubiquitin ligases, termed UBR1-UBR7, that contain the ~70-residue UBR box, a motif important for the targeting of N-end rule substrates. In this pathway, specific N-terminal residues of substrates are recognized as degradation signals by UBR box-containing E3s that include UBR1, UBR2, UBR4 and UBR5. The other E3s of this set, UBR3, UBR6 and UBR7, remained uncharacterized. Here we describe the cloning and analyses of mouse UBR3. The similarities of UBR3 to the UBR1 and UBR2 E3s of the N-end rule pathway include the RING and UBR domains. We show that HR6A and HR6B, the E2 enzymes that bind to UBR1 and UBR2, also interact with UBR3. However, in contrast to UBR1 and UBR2, UBR3 does not recognize N-end rule substrates. We also constructed UBR3-lacking mouse strains. In the 129SvImJ background, *UBR3*$^{-/-}$ mice died during embryogenesis, whereas the C57BL/6-background *UBR3*$^{-/-}$ mice exhibited neonatal lethality and suckling impairment that could be partially rescued by litter size reduction. The adult *UBR3*$^{-/-}$ mice had female-specific behavioral anosmia. Cells of the olfactory pathway were found to express β-galactosidase (LacZ) that marked the deletion/disruption *UBR3*$^{-}$ allele. The *UBR3*-specific LacZ expression was also prominent in cells of the touch, vision, hearing and taste systems, suggesting a regulatory role of UBR3 in sensory pathways, including olfaction. By analogy with functions of the UBR domain in the N-end rule pathway, we propose that the UBR box of UBR3 may recognize small compounds that modulate the targeting, by this E3, of its currently unknown substrates.

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References

Kwon, Y.T., Reiss, Y., Fried, V.A., Hershko, A., Yoon, J.K., Gonda, D.K., Sangan, K., Copeland, N.C., Jenkins, N.A. and Varshavsky, A. (1998) *Proc. Natl. Acad. Sci. USA* **95**:7898-7903.

Tasaki, T., Sohr, R., Hellweg, R., Hortnagl, H., Varshavsky, A. and Kwon, Y. T. (2007) *J. Biol. Chem.* **282**:18510-18520.

459. Phosphorylation of UBR1, its regulation and functions

Cheol-Sang Hwang, Zanxian Xia, Alexander Varshavsky

S. cerevisiae UBR1, the E3 of the yeast N-end rule pathway, is phosphorylated *in vivo*, but the role(s) of this UBR1 modification in the multiple functions of the N-end rule pathway is unknown. Phosphorylation sites on UBR1 and the kinases/phosphatases involved remain to be identified, as well. We are using biochemical and genetic approaches to understand, in functional and mechanistic detail, this modification of UBR1.

460. The N-end rule pathway as a sensor of heme

9Rong-Gui Hu, Haiqing Wang, Alexander Varshavsky

Heme is an iron-containing protoporphyrin IX. Two major species of heme are ferrous (Fe^{2+}) heme and its ferric (Fe^{3+}) counterpart, called hemin. Intracellular proteins whose functions depend on their binding to heme include hemoglobins, cytochrome oxidases, NO synthases, cGMP cyclases, and catalases, as well as specific kinases, transcription factors, ion channels, and regulators of iron metabolism. A major aspect of heme is its ability to interact with physiologically relevant gases such as O_2, NO and carbon monoxide (CO). Over the last decade, it became clear that reactive oxygen species (ROS), if they are present at "signaling" (i.e., sufficiently low) levels, can act as regulators of circuits that underlie not only stress responses but other functions as well, including the cell cycle, transcription and differentiation. The proximal sensors of H_2O_2 and analogous compounds are cysteine-containing proteins, some of which contain heme as well. ROS-mediated formation of disulfides or other Cys derivatives changes the activity of sensor proteins, which transduce their altered states into the outputs of circuits they control. In some hemoproteins of these circuits, it is the heme moiety, rather than Cys residues, that functions as a redox sensor.

We discovered (Hu *et al.*, submitted for publication) that the arginyl-transferase ATE1, which mediates the arginylation branch of the N-end rule pathway (see Fig. 1 and Introduction), is inhibited by hemin (Fe^{3+}-heme), via a specific redox mechanism that involves the formation of disulfide between cysteine-71 and cysteine-72 of ATE1. Remarkably, hemin also induces degradation of arginyl-transferase *in vivo*, thus acting as both a "stoichiometric" and "catalytic" down-regulator of the N-end rule pathway. This proteolytic circuit, a known sensor of short peptides, nitric oxide and oxygen, is now a sensor of heme as well. One function of the N-end rule pathway may be to coordinate the activities of small effectors, both reacting to and controlling the redox dynamics of heme, oxygen, nitric oxide and thiols, in part through conditional degradation of specific transcription factors and G-protein regulators.

Reference

Hu, R.G., Wang, H. and Varshavsky, A. Submitted for publication.

461. Targeting the absence: Homozygous deletions as immutable signposts for cancer therapy

Alexander Varshavsky

A major obstacle to drug-based therapies of human diseases that are both efficacious and substantially free of side effects is the massive interconnectedness and redundancy of molecular circuits in living cells. In the case of cancer, the problem is exacerbated by genomic instability of many, possibly most, cancers. This property increases heterogeneity of malignant cells in the course of tumor progression or anticancer treatment, and is one reason for the failure of most drug-based cancer therapies. A few relatively rare cancers, such as testicular carcinoma, Wilm's kidney tumor, and some leukemias in children, can often be cured through chemotherapy, but require cytotoxic treatments of a kind that cause severe side effects and are themselves carcinogenic. Several recent advances, including the use of antiangiogenic compounds and inhibitors of specific kinases, hold the promise of efficacious, curative therapies. Nevertheless, major human cancers are still incurable once they have metastasized.

I recently suggested a new approach to cancer therapy. It involves homozygous deletions (HDs). Previous studies have demonstrated that many human cancers, including major ones, contain a significant number of scattered homozygous deletions. A salient property of an HD that involves DNA sequences not present elsewhere in the genome is that HD cannot revert. Employing homozygous deletions – *not their effects on tumor suppression and RNA/protein circuits but deletions themselves* – as a target of therapy is a new idea, to my knowledge. The difficulty here is that HD is an "absence," and therefore, it cannot be a conventional molecular target. Nevertheless, an HD-specific anticancer regimen is feasible, through an approach termed *deletion-specific targeting* (DST).

The DST strategy (Varshavsky, 2007) is implemented by molecular circuits that combine, in a novel way, both existing and new methodologies. One of them is the ubiquitin (Ub) fusion technique (Varshavsky, 2005). In addition, an essential part of DST strategy is based on "helper-dependent" split-protein devices, introduced by Johnsson and Varshavsky (1994) with the split-Ub assay and thereafter extended to other split-protein constructs, such as dihydrofolate reductase (DHFR), green fluorescent protein (GFP), and beta-lactamase. Split-protein domains coupled to DNA-recognizing proteins (Stains *et al*., 2005) are also a component of DST strategy. Yet another part of DST is a feedback mechanism that receives input from a circuit operating as a Boolean OR gate and involves the activation of split nucleases, which destroy DST vector in normal

(non-target) cells. The DST strategy is independent, to a striking extent, of considerations that underlie other approaches to cancer therapy. For example, DST does not involve a function of deleted DNA, or its levels of expression in normal cells, or tumorigenic alterations of RNA/protein circuits in cancer cells, or cell-surface differences between them and normal cells. The logic of DST makes possible an incremental and essentially unlimited increase in the selectivity of therapy. If DST strategy can be implemented in a clinical setting, it may prove to be curative and substantially free of side effects.

At this stage, the nearest aim is not a clinically realistic DST design but rather the circuit's ability to function as a DST device. Work to verify the feasibility and medical relevance of DST is under way in our lab.

References

Johnsson, N. and Varshavsky, A. (1994) *Proc. Natl. Acad. Sci. USA* **91**:10340-10344.

Stains, C.I., Porter, J.R., Ooi, A.T., Segal, D.J. and Ghosh, I. (2005) *J. Am. Chem. Soc.* **127**:10782-10783.

Varshavsky, A. (2005) *Meth. Enzymol.* **399**:777-799.

Varshavsky, A. (2007) *Proc. Natl. Acad. Sci. USA.* In press.

Publications

An, J.Y., Seo, J.W., Tasaki, T., Lee, M.J., Varshavsky, A. and Kwon, Y.T. (2006) Impaired neurogenesis and cardiovascular development in mice lacking the E3 ubiquitin ligases UBR1 and UBR2 of the N-end rule pathway. *Proc. Natl. Acad. Sci. USA* **103**:6212-6217.

Connor, R.E., Piatkov, K.P., Varshavsky, A. and Tirrell, D.A. Enzymatic N-terminal addition of noncanonical amino acids to peptides and proteins. Submitted for publication.

Graciet, E. Hu, R.G., Piatkov, K., Rhee, J.H., Schwarz, E.M. and Varshavsky, A. (2006) Aminoacyl-transferases and the N-end rule pathway of prokaryotic/eukaryotic specificity in a human pathogen. *Proc. Natl. Acad. Sci. USA* **103**:3078-3083.

Hu, R.-G., Brower, C.S., Wang, H., Davydov, I.V, Sheng, J., Zhou, J., Kwon, Y.T. and Varshavsky, A. (2006) Arginyl-transferase, its specificity, putative substrates, bidirectional promoter, and splicing-derived isoforms. *J. Biol. Chem.* **281**:32559-32573.

Hu, R.G., Wang, H. and Varshavsky, A. The N-end rule pathway as a sensor of heme. Submitted for publication.

Schnupf, P., Zhou, J., Varshavsky, A. and Portnoy, D.A. (2007) Listeriolysin O secreted by *Listeria monocytogenes* into the host cell cytosol is degraded by the N-end rule pathway. *Infect. & Immun.*. In press.

Varshavsky, A. (2006) The early history of the ubiquitin field. *Protein Sci.* **15**:647-654.

Varshavsky, A. (2007) Discovering the RNA double helix and hybridization. *Cell* **127**:1295-1297.

Varshavsky, A. (2007) Targeting the absence: homozygous deletions as immutable signposts for cancer therapy. *Proc. Natl. Acad. Sci. USA.* In press.

Facilities

Flow Cytometry and Cell Sorting Facility
Genetically Altered Mouse Production Facility
Millard and Muriel Jacobs Genetics and Genomics Laboratory
Monoclonal Antibody Facility
Nucleic Acid and Protein Sequence Analysis Computing Facility
Protein Expression Center
Protein Microanalytical Laboratory

Flow Cytometry And Cell Sorting Facility
Supervising Faculty Member: Ellen V. Rothenberg
Facility Manager: Rochelle Diamond
Operators/Technical Specialists: Rochelle Diamond, Patrick Koen, Diana Perez

The Caltech Flow Cytometry and Cell Sorting Facility is located in 020 and 026 Kerckhoff Biology Building. This is a multi-user facility which provides expert assistance and advanced instrumentation to researchers for analyzing and separating various types of cells and micro organisms according to their measurable properties of light scatter and fluorescence. The power of this technology resides in its ability to analyze at high speed, light scatter and fluorescent properties of heterogeneous populations of suspended particles such as cells, microorganisms, and/or beads. These measurements provide qualitative and quantitative measurements. Statistical population characteristics for each population are revealed for all components of the sample. In addition, the particles can then be sterilely sorted based on those characteristics into tubes or multi-well plates of several configurations. Fluorescence can be used as a quantitative indicator of many different molecular and cellular properties and thus a multi-faceted tool to study many aspects of cell biology including cell cycle and proliferation, physiological function, and membrane antigen phenotype. This has innumerable applications for direct use in immunological, neurobiological, developmental, and microbiological systems to define and populations of interest. Examples are isolation of cell transfectants utilizing reporter gene expression, quantization of cell death, single-cell cloning by direct cell deposition, isolation of populations for preparation of cDNA for qPCR and gene chip analysis.

The facility is equipped with two research grade flow cytometer cell sorters and one analyzer:

- BD FACSAria, capable of analyzing at least nine colors utilizing three lasers (407 nm, 488 nm, and 633 nm), and of carrying out four-way sorting up to 10,000 cells per second with reliable efficiency and recovery, or one-way sorting, such as for single-cell cloning, into various cell culture plate configurations.
- BD FACSVantage SE, capable of analyzing five colors using two lasers, and of two-way sorting up to 3,000 cells per second or one-way sorting into various cell culture plate configurations.
- BD FACSCalibur, a four-color analyzer, together with an offline workstation, which are available to researchers for self-service analysis provided that they demonstrate competence to use the instrument or take training provided by the facility.
- The facility makes Treestar's Flowjo off-line analysis program available to its clients through a network license.
- The facility provides consultation services to all researchers on issues relating to flow cytometry, cell sorting, and cell separation techniques.

This past year the facility serviced 28 laboratories from the Biology, Chemistry, Chemical Engineering, Applied Physics, and Geobiology divisions as well as labs from USC, CSULA, and JPL. In all, the projects of 60 individual users were serviced.

The facility has provided continuing support for many laboratories with established applications using multicolor analysis of cell surface phenotype and sensitive high-speed sorting or cloning of mammalian cells. However, one of the strengths of the facility is its flexibility for new applications. In the past year, some of the more novel projects include the following:

Kimberly Beatty of the Tirrell Group has been visualizing a subset of the proteome inside cells utilizing the bioorthogonal ligation of reactive non-canonical amino acids with fluorogenic dyes. Newly synthesized proteins can be distinguished by the incorporation in their sequence of a uniquely reactive amino acid analogue from all pre-existing proteins, which are composed of the twenty natural amino acids. The reactive analogue is then utilized to tag proteins with a fluorescent dye using a highly selective azide-alkyne ligation. For the Tirrell group, the BD Bioscience FACSAria flow cytometer has been used to quantify the dye abeling of both live and fixed mouse embryonic fibroblasts. The high throughput nature of this machine has made it possible to rapidly select optimal dye concentrations and labeling lengths for proteomic visualization.

Stephanie Culler of the Smolke Group has been developing a system to examine the properties of specific intronic splice silencers and enhancers in an *in vitro* cell culture system to model cancer. She has generated mini-gene constructs which when transfected into these cancer cells fluoresce green using GFP as a reporter genetically fused to her construct gene of interest. The silencers and enhancers regulate the gene thereby changing the intensity of the GFP expression. She has been able to generate statistical analyses of these regulatory events. In addition she has generated a random library of DNA mutational inserts and the facility is currently sorting selected stage-specific molecular candidates of interest.

The Patterson laboratory has been utilizing the facility to look at apoptic events in tumor cells treated with various chemotherapeutic agents to understand the mechanism of action of these drugs, using annexin V/PI staining, as well as CFSE cell labeling to visualize cell proliferation doublings in the treated cells.

Carol Readhead of the Fraser Group has been analyzing and sorting different populations of germline stem cells in both the embryo and the adult mouse expressing GFP under the Oct4 promoter in order to study male germ cells in the embryo, neonate and adult. FACS analysis of these cells with other markers shows the germline stem cells to be a heterogeneous population that changes during different stages of development. These changes correlate with changing cell behaviors as measured by *ex vivo* confocal videomicroscopy. She has also been analyzing and sorting pig sperm.

Doug Yung from the Ponce lab at JPL used the facility to analyze microbial content in precious Arctic and Greenland ice core samples, to provide cell counts and viable fraction information. Our facility consulted with U. of Pennsylvania on the technique to perform this rare protocol.

Sagar Damle of the Eric Davidson group has been using the facility to determine the mechanism of an experimental cell fate alteration in sea urchin embryos. He has been sorting very small numbers (<400 cells) of sea urchin embryo cells derived from eggs microinjected with BAC-sized DNA fragments encoding GFP and the transcription factor Gcm under the control of a heterologous regulatory system. This system causes fate conversion of the cells that are forced to express Gcm. By taking the cells at various developmental timepoints he can measure the long-term effects of redirected Gcm expression in primary mesenchyme cells by qPCR of various transcription factor genes in the GFP^+ cells.

Publications in the past year that were based on work performed in the Facility include:

Beatty, K.E., Liu, J.C., Xie, F., Dieterich, D.C., Schuman, E.M., Wang, Q. and Tirrell, D.A. (2006) Fluorescence visualization of newly synthesized proteins in mammalian cells. *Angewandte Chemie-International Edition* **45**(44):7364-7367.

Bartlett, D.W. and Davis, M.E. Effect of siRNA nuclease stability on the *in vitro* and *in vivo* kinetics of siRNA-mediated gene silencing. *Biotechnol. Bioeng.* Published online: 8 Dec 2006.

Budd, M.E., Reis, C.C., Smith, S., Myung, K. and Campbell, J.L. (2006) Evidence suggesting that Pif1 helicase functions in DNA replication with the Dna2 helicase/nuclease and DNA polymerase delta. *Mol. Cell Biol.* **7**:2490-500.

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C.J., Gronostajski, R.M. and Anderson, D.J. (2006) The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52**:953-968.

Franco, C.B., Scripture-Adams, D.D., Proekt, I., Taghon, T., Weiss, A.H., Yui, M.A., Adams, S.L., Diamond, R.A. and Rothenberg, E.V. (2006) Notch/Delta signaling constrains reengineering of pro-T cells by PU.1. *Proc. Natl. Acad. Sci. USA* **8**:103(32):11993-8. Epub 2006 Jul 31. Erratum in: *Proc. Natl. Acad. Sci. USA.* 2006 Sept 19;103(38):14255.

Ghaboosi, N. and Deshaies, R.J. (2007) A conditional yeast e1 mutant blocks the ubiquitin-proteasome pathway and reveals a role for ubiquitin conjugates in targeting rad23 to the proteasome. *Mol. Biol. Cell* **18**(5):1953-1963.

Hajek, P., Chomyn, A. and Attardi, G. (2007) Identification of a novel mitochondrial complex containing mitofusin 2 and stomatin-like protein 2. *J. Biol. Chem.* **23**:282(8):5670-5681.

Hawkins, K.M. and Smolke, C.D. (2006) The regulatory roles of the galactose permease and kinase in the induction response of the GAL network in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **12**;281(19):13485-92. Epub 2006 Mar 7.

Mukouyama, Y.S., Deneen, B., Lukaszewicz, A., Novitch, B.G., Wichterle, H., Jessell, T.M. and Anderson, D.J. (2006) Olig2+ neuroepithelial motoneuron progenitors are not multipotent stem cells *in vivo*. *Proc. Natl. Acad. Sci. USA* **03**:1551-1556.

Puckett, C.A. and Barton, J.K. (2007) Methods to explore cellular uptake of ruthenium complexes. *J. Am. Chem. Soc.* **129**(1):46-47.

Shin, K.J., Wall, E.A., Zavzavadjian, J.R., Santat, L.A., Liu, J., Hwang, J.I., Rebres, R., Roach, T., Seaman, W., Simon, M.I. and Fraser, I.D. (2006) A single lentiviral vector platform for microRNA-based conditional RNA interference and coordinated transgene expression. *Proc. Natl. Acad. Sci. USA* **103**(37):13759-13764. Epub 2006 Aug 31.

Tesar, D.B., Tiangco, N.E. and Bjorkman, P.J. (2006) Ligand valency affects transcytosis, recycling and intracellular trafficking mediated by the neonatal Fc receptor. *Traffic* **9**:1127-1142.

Tydell, C.C., David-Fung, E.S., Moore, J.E., Rowen, L., Taghon, T. and Rothenberg, E.V. (2007) Molecular dissection of pre-thymic progenitor entry into the T lymphocyte developmental pathway. *J. Immunol.* In press.

Yang, L., Bailey, L., Baltimore, D. and Wang, P. (2006) Targeting lentiviral vectors to specific cell types *in vivo*. *Proc. Natl. Acad. Sci. USA* **103**(31):11479-11484.

Genetically Engineered Mouse Production Facility
Director and Member of the Professional Staff:
Shirley Pease
Cryopreservation and Microinjection: Juan Silva
Embryonic Stem Cell Culture: Jue Jade Wang
Mouse Colony Manager: Jennifer Alex

In June 2005, the Genetically Altered Mouse Core and the Office of Laboratory Animal Resources (OLAR) combined to form the Caltech Laboratory Animal Services (CLAS). CLAS consists of two subdivisions, OLAR, which is headed by Dr. Janet Baer and Genetically Engineered Mouse Services (GEMs) that is headed by Shirley Pease. The purpose of the merger was to refine, streamline and standardize procedures for laboratory animal care and use on campus. GEMs continues to provide microinjection, cryopreservation, re-derivation and tissue culture services. In addition, we offer services in the form of rodent colony management and use, where required, in all animal Facilities.

Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg (Gordon *et al.*, 1980). This is a non-targeted event. Targeted disruption of specific genes, however, requires the manipulation of pluripotent embryonic stem (ES) cells *in vitro* and their subsequent return to the embryonic environment for incorporation into the developing embryo (Zijlstra *et al.*, 1989). The resulting chimeric mouse born is useful for two purposes: 1) it is comprised of tissue from two sources, the host embryo and the manipulated stem cells. More importantly, 2) it can be mated to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. (The Nobel Prize in Physiology or Medicine was awarded this year to the pioneers of this technology, Mario Capecchi, Martin Evans and Oliver Smithies). The facility, in collaboration with Anderson, Baltimore, Kennedy, Lester, Patterson, Rothenberg, Simon, Varshavsky and Wold laboratories, has generated multiple transgenic, knockout and knockin mouse strains, amounting to nearly 170 mouse strains. The Facility together with the Baltimore lab, participated in the development of a new method for the introduction of DNA into early-stage embryos (Lois *et al.*, 2002). This method makes use of non-recombinant lentivirus as a vector for the introduction of DNA into one-cell embryos. The method has proven to be highly efficient and promises to be useful for studies in mice and rats, where large numbers of constructs need to be tested. This new methodology also makes feasible the generation of transgenic animals in species that were hitherto impractical to work with, due to the very low numbers of embryos available for use. Since the lentiviral vector method was established, 79 transient or established mouse models have been generated by this means, together with one Tg rat model. Facility staff has performed all embryo manipulation involved in the production of these new lines.

Microinjection equipment has been set up within the mouse facility, which operates on restricted access as part of a barrier system designed to safeguard the microbiological status of the animals. A room outside the facility has been allocated by the Division to be used primarily for teaching graduate students, technicians and postdocs the techniques involved in transgenic mouse production. This room has been operating since July 1996. Investigators have the option of using this room to perform their own microinjection of embryos, rather than using the full technical service available from the Genetically Engineered Mouse services.

At the merger, a new position was created, that of Colony Manager. An experienced technician has been appointed to the post and is available now to assist investigators with all colony management questions, primarily assisting investigators in making sure their experimental needs are met as economically as possible. GEMs Facility staff are currently working with IMSS to develop software that will assist technicians and investigators in the management of their mice. Amongst its features, this inter-relational system will track the breeding history of each strain and have the ability to generate family trees. The system will also report on production levels for each strain. Users will access the system to enter genotype results and work requests. An electronic signal will be sent to CLAS staff when work requests are made, helping us to manage work requests in a timely manner. The system will be basic but easy to use. We anticipate this will be a very useful animal management tool.

In tissue culture and the use of embryonic stem (ES) cells, the Facility has, in the past, participated in the derivation of new ES cell lines derived from genetically altered mice (see Simon laboratory Annual Report, 2001). This year, the Facility generated over forty new and as yet untested, embryonic stem cell lines, the majority of which are from C57BL/6 mice. This was a by-product of our wish to determine the most efficient approach to deriving such cell lines, since we anticipate that investigators may wish to use ES cells derived from their own genetically altered strains of mouse. Indeed, five such new ES cell lines have been derived from for the Rothenberg lab. Several investigators are using these pluripotent cells in research that involves pushing the cells down specific developmental pathways, and also to investigate the incorporation of extraordinarily large pieces of DNA into the mouse genome. The Facility is able to offer investigators a choice between working with ES cells on a 129 background, a C57BL/6 background or an F1 background, which is a mix between these two strains. We are able to manipulate and obtain germline transmission from all these ES cell types. C57BL/6 ES cells provide a significant advantage in that the mutation will be established initially on this well understood genetic background, instead of undertaking a two-year breeding program to reach the same point, having initially established the mutation on a sub-optimal genetic background. Hybrid ES cells have been reported to be useful for their vigor. Unlike ES cells from an inbred background, (e.g., C57BL/6 and 129), it is possible to

derive from hybrid ES cells live pups that are wholly of ES cell origin. Tetraploid embryos, (embryos with twice the normal number of chromosomes), are able to develop and contribute to extra-embryonic membrane cell lineages, but **not** to the development of any fetal tissues. Thus, a tetraploid embryo at blastocyst stage, injected with hybrid ES cells, will result in the production of an animal that is wholly of ES cell origin. ES cells from inbred strains such as C57BL/6 or 129 require a contribution to the developing fetus from the injected host blastocyst itself, for the production of viable pups. We recently established the production and use of tetraploid embryos at Caltech and have our first pups born from their use in combination with hybrid ES cells.

Once a new mouse model has been characterized, it may be cryopreserved, or sent to the Mutant Mouse Resource Center, to be made available to the research community in general. We currently have 93 mouse models cryopreserved. For each line, between 200 and 500 embryos at eight-cell stage have been preserved in liquid nitrogen. There are currently 27,419 embryos frozen in total. We shall continue to preserve embryos from mouse strains. The advantages of such a resource are many. Unique and valuable mouse strains that are currently not in use may be stored economically. In the event that genetic drift should affect any strain, over time, then the option to return to the original documented genetic material is available. Lastly, in the event of a microbiological or genetic contamination occurring within the mouse facility, we have the resources to set up clean and genetically reliable mouse stocks in an alternative location.

During 2006, Facility staff received training on the culture of human embryonic stem cells. We are about to establish the culture of H1 and H9 hES cells at Caltech. Initially, our goal will be to expand the cells and to cryopreserve stocks that may be made available to Caltech investigators at a later date. There are already a few investigators in the Biology Division who will wish to use the cells as soon as they can be made available.

Presently, thirteen principal investigators and their postdoctoral fellows or graduate students use GEMs services. In addition to the maintenance of nearly 100 different targeted and non-targeted strains, we also maintain colonies of inbred and outbred animals, which are used to support the development of new lines, by investigators at Caltech. We also have many mouse models on both an inbred and an outbred background, plus intercrosses between two or three different, but related, mouse models. In total, we currently maintain nearly 200 separate strains of mouse. Some of these strains are immune-deficient and require specialized care to protect them from bacteria commonly present in immune-competent animals. In immune-deficient animals, these hitherto harmless organisms can cause a problem. This may interfere with the well being of the animal and investigator ability to obtain reliable experimental results.

Listed below are the names of the eleven principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

David Anderson
Ben Deneen, Wulf Haubensak, Christian Hochstim, Walter Lerchner, Li Ching Lo, Agnes Lukaszewic, Sophia Vrontu

David Baltimore
Mark Boldin, Shengli Hao, Lili Yang

Mark Davis - (Chemistry and Chemical Engineering)
Derek Bartlett, Eric Kowel

Ray Deshaies
Narimon Harnapour

Michael Elowitz
Fred Tan

Scott Fraser
David Koos, Carol Readhead, Nicholas Plachta

Mary Kennedy
Eduardo Marcora, Andrew Medina-Marino, Leslie Schenker, Laurie Washburn

Henry Lester
Purnima Deshpande, Princess Imoukhuede, Herwig Just, Raad Nashmi

Paul Patterson
Ben Deverman, Ali Koshnan, Natalia Malkova, Limin Shi, Stephen Smith

Ellen Rothenberg
Deirdre Scripture-Adams, Chase Tydell, Mary Yui, Mark Zarnegar

Melvin Simon
Valeria Mancino

Alexander Varshavsky
Christopher Brower, Jun Sheng

Barbara Wold
Brian Williams

References

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H. (1980) *Proc. Natl. Acad. Sci. USA* **77**(12):7380-7384.

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) *Science* **295**:868-872.

Zijlstra, M., Li, E., Sajjadi, F., Subramani, S. and Jaenisch, R. (1989) *Nature* **342**(6248):435-438.

Publication

Pease, S. (2006) Ancillary Techniques. In: *Mammalian and Avian Transgenesis - New Approaches*. S. Pease and C. Lois (Eds), Springer of Berlin, Heidelberg and New York, pp. 231-276.

Millard and Muriel Jacobs Genetics and Genomics Laboratory

Director: José Luis Riechmann

Staff: Brandon King, Vijaya Rao, Lorian Schaeffer, Joanne Tan-Cabugao

Support: The work described in the following research reports has been supported by:

Millard and Muriel Jacobs Family Foundation

National Science Foundation

Summary: The goal of the Millard and Muriel Jacobs Genetics and Genomics Laboratory, in the Division of Biology, is to provide a suite of cutting-edge genomic research tools to all interested Caltech scientists, with an emphasis on large-scale gene expression profiling. The Laboratory performs gene expression analyses using DNA microarray technology, and is equipped with the necessary experimental and bioinformatics infrastructure that is needed to generate, store, and analyze large-scale datasets from a variety of microarray technological platforms. During the period of this report, the Laboratory has continued to provide support for genomics research to the Caltech community at large, including groups from the Division of Biology, the Division of Chemistry and Chemical Engineering, and the Division of Engineering and Applied Science.

In addition to the broad mission of the Laboratory, we are interested in the analysis of regulatory networks in *Arabidopsis* using genomic technologies, in particular those networks that are related to flower development. An important class of regulatory molecules in animals and plants are microRNAs (miRNAs): small non-coding RNAs (~20-24 nt in length) that regulate gene expression in a sequence-specific manner by targeting mRNAs for cleavage or translational repression. We are using genomic technologies (such as DNA microarrays) to characterize the *Arabidopsis* complement of microRNAs and its participation in floral development processes.

Research Support

Division of Biology

The Laboratory has worked with the groups of Professors David Anderson, David Baltimore, Seymour Benzer, Eric Davidson, Elliot Meyerowitz, Paul Patterson, Paul Sternberg, Alex Varshavsky, and Barbara Wold. Gene expression experiments have been performed using various technologies (Affymetrix, in-house manufactured microarrays), on various organisms (human, mice, *Drosophila*, *C. elegans*, *Arabidopsis*, *E. coli*), and for a wide variety of research projects. These include: transcription factors controlling mammalian neural stem cell development (Anderson); signal transduction, transcriptional regulation, and microRNAs in the immune system (Baltimore); flower development in *Arabidopsis* (Meyerowitz); interactions between the nervous and immune systems (Patterson); molecular genetics of nematode development and behavior (Sternberg); and the N-end rule protein degradation pathway (Varshavsky), among others.

Division of Chemistry and Chemical Engineering

The Laboratory has worked with the groups of Professors Linda Hsieh-Wilson, and Peter Dervan. The Laboratory has manufactured carbohydrate microarrays that the Hsieh-Wilson group has used in its research, and performed gene expression profiling experiments using the Affymetrix technology for Professor Peter Dervan′s laboratory.

Division of Engineering and Applied Science

The Laboratory has worked together with the group of Professor Babak Hassibi to develop a real-time microarray technology that allows for the detection of interactions between probes and targets as the reaction takes place. The technology offers improvements in sensitivity, accuracy, and dynamic range over the existing microarray platforms.

Infrastructure and capabilities

Since inception, the Laboratory has been well equipped to manufacture microarrays and to perform gene expression analyses using various microarray platforms, including Affymetrix GeneChips. Available equipment in the laboratory includes a MicroGrid II arrayer (BioRobotics), a GenePix 4200A scanner (Molecular Devices/Axon Instruments), a BioAnalyzer (Agilent Technologies), a MAUI hybridization system (Biomicrosystems), a QIAGEN 3000 liquid handling robot, an Affymetrix GeneArray 3000 7G scanner and fluidics station, and a high-throughput real-time PCR system (LightCycler 480, from Roche). A more comprehensive description of the laboratory infrastructure is available at http://mmjggl.caltech.edu/

The MicroGrid II arrayer allows us to produce large numbers of microarray slides in a cost-effective manner, and to produce customized microarrays for which there is no equivalent commercial counterpart available. For example, we produce whole genome *Arabidopsis* microarrays using 70-mer oligonucleotide sets from Operon, which are used in various research projects related to flower development (work performed together with Professor E.M. Meyerowitz′s group). Custom carbohydrate microarrays are used to study glycosaminoglycan-protein interactions (Professor Linda Hsieh-Wilson, Division of Chemistry). MicroRNA microarrays have been produced for mouse (Professor David Baltimore) and *Arabidopsis* (see below). Other customized microarrays include microarrays printed with Cy3-labeled probes, which are used to study the dynamics of DNA hybridization in real time (Professor Babak Hassibi, Division of Engineering and Applied Science).

The laboratory uses Resolver (from Rosetta Biosoftware) as its primary gene expression data analysis system. Resolver is a robust, enterprise-scale, gene expression system that combines a high capacity database and advanced analysis software in a high-performance server framework. The system is accessible through client

stations using a web-based interface. We also have at our disposal additional microarray software tools and analysis packages, both public and commercial. The hardware infrastructure of the laboratory currently includes a Sun Fire V880 server (from Sun Microsystems), that we use for the Resolver database (Oracle) and analysis system.

To expand capabilities, a new instrument was recently added to the Laboratory: a next-generation sequencer, the Illumina Genome Analyzer. The Genome Analyzer uses a massively parallel sequencing approach to generate short sequence reads with ultra-high throughput (more than one billion bases of data in a single run) and reduced cost.

462. Genomic analyses of *Arabidopsis* miRNAs: their roles in flower development

Brandon King, Vijaya Rao, Lorian Schaeffer, Yuling Jiao[1], José Luis Riechmann

The focus of this project is to characterize the functions of the *Arabidopsis* complement of microRNAs (miRNAs), and to identify the gene regulatory networks in which they may participate, in particular, during flower development, by using microarray analysis of miRNA expression and other genomic approaches. MicroRNAs are small non-coding RNAs that regulate gene expression in a sequence-specific manner, and they have emerged as a very important class of regulatory molecules in plants and in metazoans. In *Arabidopsis*, more than 100 miRNAs have already been detected and/or predicted, and the *Arabidopsis* genome may, in fact, contain several hundred distinct miRNA loci, a number that demands the development of high-throughput methodologies for their study.

We have completed our analysis of various *Arabidopsis* miRNA computational predictions and of small RNA sequence sets, and selected the sequences to be represented on our miRNA microarray. For those sequences, microarray probes have been designed using a customized design pipeline. As a result, we have a collection of 2382 probes, encompassing: all known *Arabidopsis* miRNAs, a filtered selection of computationally predicted miRNAs, and a filtered selection of small RNA sequences derived from deep-sequencing projects or from various small-scale projects (as well as all known miRNA sequences from *C. elegans* and *Drosophila*, to facilitate experimental design and data processing). We manufacture our microarrays by contact printing, and we have vetted the resulting miRNA microarray slides in self-to-self and differential expression experiments. Using these microarrays, we are conducting experiments to determine the gene expression patterns of the *Arabidopsis* complement of miRNA genes during the process of flower development.

In addition, to detect potential miRNA targets in a comprehensive manner, we are using an experimental approach that combines the use of specific mutant backgrounds, our standard whole-genome microarrays, and a method to isolate RNA that is specifically enriched for decapped and 5′ phosphorylated mRNAs.

[1]*Postdoctoral Scholar, Caltech (Meyerowitz laboratory)*

Publications

Kannangara, R., Branigan, C., Liu, Y., Penfield, T., Rao, V., Mouille, G., Höfte, H., Pauly, M., Riechmann, J.L. and Broun, P. (2007) Transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis*. *Plant Cell* **19**(3):1278-1294.

Riechmann, J.L. (2007) Transcription factors of *Arabidopsis* and rice: A genomic perspective. In: *Regulation of Transcription in Plants*, pp28-53, K.D. Grasser (ed.), Annual Plant Reviews, Volume 29, Blackwell Publishing, Oxford.

Sieber, P., Wellmer, F., Gheyselinck, J., Riechmann, J.L. and Meyerowitz, E.M. (2007) Redundancy and specialization among plant microRNAs: Role of the *MIR164* family in developmental robustness. *Development* **134**:1051-1060.

Wellmer, F., Alves-Ferreira, M., Dubois, A., Riechmann, J.L. and Meyerowitz, E.M. (2006) Genome-wide analysis of gene expression during early *Arabidopsis* flower development. *PLoS Genetics* **2**(7), e117.

Publications acknowledging the laboratory

Burnett, R., Melander, C., Puckett, J.W., Son, L.S., Wells, R.D., Dervan, P.B. and Gottesfeld, J.M. (2006) DNA sequence-specific polyamides alleviate transcription inhibition associated with long GAA.TTC repeats in Friedreich's ataxia. *Proc. Natl. Acad. Sci. USA* **103**(31):11497-11502.

Deneen, B., Ho, R., Lukaszewicz, A., Hochstim, C.J., Gronostajski, R.M. and Anderson, D.J. (2006) The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52**(6):953-968.

Gama, C.I., Tully, S.E., Sotogaku, N., Clark, P.M., Rawat, M., Vaidehi, N., Goddard, W.A., 3rd., Nishi, A. and Hsieh-Wilson, L.C. (2006) Sulfation patterns of glycosaminoglycans encode molecular recognition and activity. *Nat. Chem. Biol.* **2**(9):467-473.

O'Connell, R.M., Taganov, K.D., Boldin, M.P., Cheng, G. and Baltimore, D. (2007) MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. USA* **104**(5):1604-1609.

Shipp, E.L. and Hsieh-Wilson, L.C. (2007) Profiling the sulfation specificities of glycosaminoglycan interactions with growth factors and chemotactic proteins using microarrays. *Chem. Biol.* **14**(2):195-208.

Taganov, K.D., Boldin, M.P., Chang, K.J. and Baltimore, D. (2006) NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. USA* **103**(33):12481-12486.

Monoclonal Antibody Facility
Supervisor: Paul H. Patterson
Director: Susan Ker-hwa Ou
Staff: Shi-Ying Kou

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (mAbs), ascites fluid or other related tissue culture services. We also produce polyclonal ascites Abs by immunizing mice with antigen and then inducing the mice with sarcoma cells to obtain high titer, polyclonal ascites fluid. This method can provide 10-18 ml polyclonal ascites fluid per mouse by using small amounts of antigen. In addition to these service functions, the Facility also conducts research on the development of novel immunological techniques.

In its service capacity, the Facility produced Abs for the following groups during the past year. The Zhukovsky lab (Xencor) obtained mAbs against human BAFF, a type II membrane protein that promotes the proliferation and survival of activated B cells by inducing expression of pro-survival oncogenes. The Hill lab (UCLA) obtained mAbs against Gas-11, which represents the mammalian counterpart of Trypanin, which is produced by various species of trypanosomes. The Bjorkman lab obtained polyclonal ascites fluid against the Fc receptor for chicken immunoglobulin (IgY). The Hay lab obtained polyclonal antiserum against a peptide from *Drosophila* protein Relish-C. The Zinn Lab obtained polyclonal ascites fluid against the 3rd exon of a leucine-rich cell adhesion molecule from *Drosophila*, and polyclonal ascites fluid against the transmembrane domain of *Drosophila* G protein receptor. The Strauss lab obtained polyclonal ascites fluid against the viral envelope (E) protein of yellow fever virus, and polyclonal ascites fluid against the NS-5 protein from West Nile virus. The Strauss lab also obtained polyclonal ascites fluid against the dimer and dodecamer of NS1 protein from dengue virus, and polyclonal ascites fluid against NS3 and NS3 helicase protein from dengue 2 virus.

We are currently working with the following groups: The Zinn lab is trying to obtain polyclonal ascites fluid against a gene encoding a *zona pellucida* domain that is expressed in various epithelial tissue during *Drosophila* embryogenesis, as well as polyclonal ascites fluid against polyA-binding protein (PABP), and polyclonal ascites fluid against Nanos (a translation repression factor). The Strauss lab is trying to obtain polyclonal ascites fluid against the capsid protein of West Nile virus and the NS5 protein from dengue 2 virus. The Schuman lab is trying to obtain polyclonal ascites fluid against POMP (proteasome maturation protein). The Ou lab (USC) is trying to obtain mAbs against hepatitis C virus F protein. The Patterson lab is trying to obtain polyclonal ascites fluid against mutant huntingtin exon 1.

Publications that were based on the work done by the Facility

Baron, D.M., Kabutatu, Z.P. and Hill, K.L. (2007) Stuck in reverse: Loss of LC1 in *Trypanosome brucei* disrupts outer dynein arms and leads to reverse flagellar blat and backward movement. *J. Cell. Sci.* **120**:1513-1520.

Devin, B.T., Tiangco, N.E. and Bjorkman, P.J. (2006) Ligand valency affects transcytosis, recycling and intracellular trafficking mediated by the neonatal Fc receptor. *Traffic* **7**:1127-1142.

Hong, E., Perera, R., and Kuhn, R.J. (2006) Alphavirus capsid protein helix I controls a checkpoint in nucelocapsid core assembly. *J. Virol*. **80**:8848-8855.

Maslov, D.A., Spremulli, L.L., Sharma, M.R., Bhargava, K., Grasso, D., Falick, A.M., Agrawal, R.K., Parker, C.E. and Simpson, L. (2007) Proteomics and electron microscopic characterization of the unusual mitochondrial ribosome-related 45S complex in *Leishmania tarentolae*. *Mol. Biochem. Parasitol.* **152**:203-212.

Pinkett H.W., Lee, A.T., Lum, P., Locher, K.P. and Rees, D.C. (2007) An inward-facing conformation of a putative metal-chelate-type ABC transporter. *Science* **315**:373-377.

Rees, D.C., Lee, A.T., Locher, K.P. and Pinkett, H.W. (2006) The structural studies of ABC transporters. *FASEB J.* **20**:A1338.

Tully, S.E., Rawat, M. and Hsieh-Wilson, L.C. (2006) Discovery of a TNF-alpha antagonist using chondroitin sulfate microarrays. *Am. Chem. Soc.* **128**:7740-7741.

Nucleic Acid and Protein Sequence Analysis Computing Facility
Supervisor: Stephen L. Mayo
Staff: David R. Mathog

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Current SAF hardware consists of a Sun Netra running Solaris, a small 20-node Beowulf cluster, a file server, a 26 ppm duplexing laser printer, and a 16 ppm duplexing color laser printer. The PCs that comprise the "structure analysis facility" are also located in our facility.

Most common programs for sequence analysis are available on the primary server http://saf.bio.caltech.edu/. These include the GCG and EMBOSS Packages, PRIMER3, Phred, Phrap, Cross_Match, Phylip, and HMMER. Many of these may be accessed through the W2H or Pise web interfaces. Other programs, custom written programs, or special databases are available on request. The PCs support hardware stereo under both Linux and Windows. Under Linux the programs Coot, O, Molscript, XtalView, CCP4, and Delphi are available. Under Windows Wincoot, Swiss PDB Viewer, O, PyMol, POVray, and various drawing and animation programs may be used. The searchable documentation for these programs is available on the SAF web server. The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are also available on the SAF web server. Web pages have been provided for running common compute intensive jobs locally on the SAF Beowulf cluster. BLAST executes in a parallel mode so that searches complete faster than they do at the NCBI server. An enhanced parallel HHMER server offers the full set of HMMER programs plus the unique ability to search any of the installed BLAST databases with an HMM. Personal BLAST sequence databases up to 50Mb may be uploaded and searched. The multiple sequence alignment programs T-COFFEE, POA, Probcons, MAFFT, and Muscle are also available. Traces from any DNA sequencing facility may be uploaded and analyzed. The SAF also distributes these site licensed programs for PCs and Macs: DNASTAR, Gene Construction Kit, ChemSketch, and X-Win.

Protein Expression Center
Director: Jost Vielmetter
Faculty Advisers: Pamela J. Bjorkman, Mary B. Kennedy
Supervisor: Barbara J. Wold
Staff: Gilberto DeSalvo, Chris Fogelsong, Inderjit Nangiana

Highlights of the year

The Protein Expression Center has continued to provide protein expression and purification services during 2007 mostly for Caltech researchers, but also for outside clients. The majority of protein production was based on Baculovirus expression culture. However, the center was able to expand the spectrum of activities significantly to provide extended services. Among the highlights were some very challenging projects that included the successful production of active DNA polymerase variants, which are notoriously difficult to produce and require highly specialized expression and purification technologies.

Another highlight was our ability to increase the production of bacterially expressed proteins significantly. Examples are active eukaryotic DNA-binding proteins and bacterial enzymes, morphogenetic factors and transcription factor fragments serving as antigens for antibody production to support for example Chromatin Immuno Precipitation (ChIP). In addition to accomplishing successful expression of these proteins, we were able to develop and implement a variety of sophisticated purification technologies that involve several dimensions of chromatography modes often in combination of up to four different techniques.

Further progress has been made in the Center by implementing protein expression optimization experiments on a routine basis to ensure optimal and efficient expression. This was critical to accomplish projects that involve simultaneous multimeric expression of difficult protein complexes such as ubiquitin ligase and ubiquitin kinase complexes. These optimization experiments require a systematic approach and a significant amount of extra time but are the only way to success with challenging protein complexes.

Another highlight is the successful implementation of small to medium scale protein production based on mammalian (human) suspension cell culture. This is a very potent key technology that allows us to produce proteins with post-translational modifications that resemble their human native counterparts more closely than proteins produced in any other system. This is absolutely critical for a number of major research projects, in particular the "Engineering Immunity" project that the Bjorkman and Baltimore labs are working on. The Center was able to build and implement the infrastructure for this important protein production technology. The goal for the future is to expand our abilities to mid and large-scale mammalian protein production by implementing "Wave" culture technology, which allows to produce proteins in 5 to 20 liter cultures. Our current suspension culture setup is a necessary prerequisite for this advanced technology.

An important additional benefit that came along with the mammalian suspension culture facility is our ability to express transient or stable "bait protein" systems that enable the production and isolation of mammalian protein complexes in their natural environment. These projects lead to potential new discoveries of participant protein subunits in these protein complexes. These can, for example, be identified by using the new Mass-Spectroscopy Center, led by Sonja Hess, and insofar this new ability dovetails perfectly with recent efforts of the Beckman Institute to create a unique infrastructure for proteomic discovery projects. We have so far successfully expressed two different protein complexes, which yielded very promising results and we look forward to continue these efforts.

Another highlight and new feature of the Protein Expression Center is the acquisition and implementation of the Biacore T100 instrument. This significant addition to the Center was made possible through Mary Kennedy and her co-applicants for the "Center for Integrative Study of Cell Regulation" Moore grant. The interest and use of this instrument has steadily increased since the acquisition of the instrument and is expected to become a very valued asset in the Caltech research community to study biomolecular interactions.

Publications acknowledging the Expression Center

Davis, M.I., Bennett, M.J., Thomas, L.M. and Bjorkman, P.J. (2005) Crystal structure of prostate-specific membrane antigen, a tumor marker and glutamate carboxypeptidase. *Proc. Natl. Acad. Sci. USA* **102**:5981-5986.

Hamburger, A.E., West, Jr. A.P. and Bjorkman, P.J. (2004) Crystal structure of a polymeric immunoglobulin-binding fragment of the human polymeric immunoglobulin receptor. *Structure* **12**:1925-1935.

Hamburger, A.E., West, Jr., A.P., Hamburger, Z.A., Hamburger, P. and Bjorkman, P.J. (2005) Crystal structure of a secreted insect ferritin reveals a symmetrical arrangement of heavy and light chains. *J. Mol. Biol.* **349**:558-569.

Luo, R., Mann, B., Lewis, W.S., Rowe, A., Heath, R., Stewart, M.L., Hamburger, A.E., Sivakolundu, S., Lacy, E.R., Bjorkman, P.J., Tuomanen, E. and Kriwacki, R.W. (2004) Solution structure of choline binding protein A, the major adhesin of *Streptococcus pneumoniae*. *EMBO J.* **24**:34-43.

Verma, R., Oania, R., Graumann, J. and Deshaies, R.J. (2004) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell* **118**:99-110.

Protein Microanalytical Facility (PPMAL)
Director: Jie Zhou
Associate Biologist: Felicia Rusnak
Faculty Advisor: Professor Mary Kennedy

Activity
Mass spectrometry of large biomolecules and small organic molecules
Proteomics (In-gel enzymatic protein digestion; LC/MS/MS and data base search)
Protein (Edman) chemical sequencing
De novo peptide sequencing by mass spectrometry

Equipment
Quadrupole time-of-flight mass spectrometer (ABI QstarXL)
Triple quadrupole mass spectrometer (MDS Sciex API 365)
MALDI-TOF mass spectrometer (ABI Voyager de.str)
Capillary protein sequencer (Procise cLC, ABI 492)
HPLC nanoflow, 2D (Eksigent)
HPLC (ABI microbore 140D pump, PE UV monitor)
MASCOT server

New Applications
Application of 2-hour-long gradient in LC/MS/MS enabled us to collect up to 400 MS/MS spectra to identify over 100 proteins from the digests of the protein mixtures pulled down from some binding columns or beads for the Varshavsky group. With our 2-dimensional LC system, we can analyze even more complicated protein mixtures. We also successfully identified low-level membrane proteins in gel bands from the Clemons lab. Very low-level proteins from silver stain gel bands were also identified for the Simon lab. The Bronner-Fraser and Benzer labs have also started to submit gel bands for protein identification. Another on-going application is to locate methylation sites of proteins for the Baltimore lab. Preliminary results were obtained with LC/MS/MS and mass difference, 14, survey scan.

Throughput and Interactions
During the first 6 months of the fiscal year 2007, PPMAL interacted with 23 laboratories. Samples were analyzed from the Division of Biology, and Chemistry and Chemical Engineering (see list). A total of 1822 samples were analyzed. In addition to our work for campus faculty and staff, work was also performed for Nanogen, UCLA, UC Berkeley and Harvard Children's Hospital (57 samples analyzed).

Mass Sectrometry
In six months, 1,700 proteins, peptides, oligonucleotides, and carbohydrates, small organic compounds have been analyzed, a 30% increase over the same period of last year. This extrapolates to an annual throughput of over 3,400. Our off-campus activity recorded 54 samples.

Proteomics
For the period covering this report, 103 digests had been analyzed. This amount is a 20% increase over the same period last year. This extrapolates to an annual throughput of 200 samples.

Protein and Peptide Sequence Analysis
The lab has sequenced proteins and peptides for 204 cycles, a 40% increase over the same period last year. The average number of residues per sample decreased from 8 to 5.

Protein Microanalytical Facility (PPMAL) October, 2006 – March, 2007 List of facility users and activity						
On-Campus						
	# Samples	**# Mass**	**# Proteomics**	**# Sequences**	**# Cycles**	**# HPLC**
Attardi	2		2			
Barton	1003	1003				
Bronner-Fraser			16			
Bjorkman	18	9		9	79	
Clemons	10		10			
Collier	7	7				
Dervan	24	24				
Dougherty	55	55				
Fraser	61	61				
Gray	143	139	4			
Grubbs	6	4	2			
Heath	56	54	2			
Hsieh-Wilson	89	89				
Mayo	61	61				
Newman	13		13			
Parker	5		5			
Peters	11	11				
Simon			2			
Rees	14		12	2	12	
Tirrell	135	90	22	23	70	
Varshavsky	28	13	12	3	15	
Winkler	26	26				
TOTALS	**1765**	**1646**	**102**	**37**	**176**	**0**
Off-Campus						
UC Berkeley			1			
Nanogen	54	54				
UCLA	3	0	0	3	28	
TOTALS	**57**	**54**	**0**	**3**	**28**	**0**

Graduates

DIVISION OF BIOLOGY
DOCTOR OF PHILOSOPHY - 2007

Oscar Alvizo, Ph.D.
Biochemistry and Molecular Biophysics
B.S., University of California, Santa Cruz, 2001
Thesis: Computational Protein Design Force Field Optimization: A Negative Design Approach

Meredith Howard Ashby, Ph.D.
Biochemistry and Molecular Biophysics
B.S., Amherst College, 1996
Thesis: The Sea Urchin Regulome in Development

Ulrik Ravnborg Beierholm, Ph.D.
Computational and Neural Systems
B.S., University of Copenhagen, 1999; M.S., 2001
Thesis: Bayesian Modeling of Sensory Cue Combinations

John Andrew Bender, Ph.D.
Biology
B.S., Montana State University, 2001
Thesis: Elements of Feed-forward and Feedback Control in *Drosophila* Body Saccades

C. Titus Brown, Ph.D.
Biology
B.A., Reed College, 1997
Thesis: Tackling the Regulatory Genome

Seth Alexander Budick, Ph.D.
Biology
B.A., Swarthmore College, 1998
Thesis: Resource Localization and Multimodal Flight Control in *Drosophila melanogaster*

Michael Campos, Ph.D.
Biology
B.S., Northwestern University, 2000
Thesis: Eye Movements and Reward, Sequential States, and Context-Dependent Target Selection

Scott A. Detmer, Ph.D.
Biology
B.S., University of California, San Diego, 1999
Thesis: The Role of Mitofusin Proteins in Mitochondrial Fusion and Disease

Jolene Sabrina Fernandes, Ph.D.
Biology
B.Sc., Suffolk University, 2000
Thesis: Dissection of Gene Regulatory Networks Underlying Patterning and Morphogenesis in the *C. elegans* Vulva

Nazli Ghaboosi, Ph.D.
Genetics
B.A., University of California, Berkeley, 1996
Thesis: Genetic Inhibition of the Ubiquitin-Proteasome Pathway: Insights into Proteasomal Targeting

Carl Gold, Ph.D.
Computation and Neural Systems
A.B., B.S., Stanford University, 1995; M.S., New York University, 1999, M.S., Kings College London, 2000
Thesis: Biophysics of Extracellular Action Potentials

Gregory Phillip Henderson, Ph.D.
Biology
B.S., University of California, Berkeley, 2000
Thesis: Ultrastructural Studies of Two Model Minimal Cells by Electron Cryotomography

Christian John Hochstim, Ph.D.
Biology
B.S., Yale University, 1999
Thesis: Pax6 Controls Astrocyte Positional Identity in the Spinal Cord

Jean Jing Huang, Ph.D.
Biology
B.A., Wellesley College, 2001
Thesis: Acyl-Homoserine Lactone Quorum Signal Degradation by Soil and Clinical *Pseudomonas sp*.

Vivek Jayaraman, Ph.D.
Computational and Neural Systems
B. Tech., Indian Institute of Technology, 1994; M.S., University of Florida, 1996
Thesis: Neural Circuit Dynamics and Ensemble Coding in the Locust and Fruit Fly Olfactory System

Jongmin Kim, Ph.D.
Biology
B.S., Pohang University, 2000
Thesis: *In Vitro* Synthetic Transcriptional Networks

Jonathan Kyle Lassila, Ph.D.
Biochemistry and Molecular Biophysics
B.A., Reed College, 1997; M.S., Yale University, 2000
Thesis: Methods for Computational Enzyme Design and Application to the Chorismate-Prephenate Rearrangement

Pei Yun Lee, Ph.D.
Biology
B.S., University of California, Los Angeles, 1999
Thesis: Function and Regulation of the *Strongylocentrotus gatae* Gene

Carole Chih-Chen Lu, Ph.D.
Biology
S.B., Massachusetts Institute of Technology, 1999
Thesis: Cranial Neural Crest Cell Migration in the Avian Embryo and the Roles of Eph-A4 and Ephrin-A5

Davin Malasarn, Ph.D.
Biology
B.S., University of California, Davis, 2000
Thesis: Molecular and Environmental Studies of Bacterial Arsenate Respiration

Jennifer Pielstick Montgomery, Ph.D.
Biology
B.A., Barnard College, 1999
Thesis: The Effects of Behavioral Stress and Endothelin Receptor Antagonists on Cancer

Farshad Moradi, Ph.D.
Computational and Neural Systems and Computer Science
M.D., Tehran; University of Medical, 2000
Thesis: Conscious Awareness Determined by Selective Gating of Information in Early Visual Areas

Eric Ardon Mosser, Ph.D.
Biology
B.S., The Evergreen State College, 1997
Thesis: Visualization of Cadherin-Cadherin Association in Living Cells

Gavin Erick Murphy, Ph.D.
Biochemistry and Molecular Biophysics
B.S., University of Dallas, 1999
Thesis: Cryoelectron Tomography of Bacteria and their Macromolecular Machines

Dylan Rhichard Nieman, Ph.D.
Computation and Neural Systems
A.B., Harvard College, 1998; M.D., University of Southern California, 1006
Thesis: Postdiction and the Effects of Spatial, Temporal, and Feature Compatibility on Sensory Integration

Kerstin Preuschoff, Ph.D.
Computation and Neural Systems
Dipl. Ing., Technical University of Berlin, 2000
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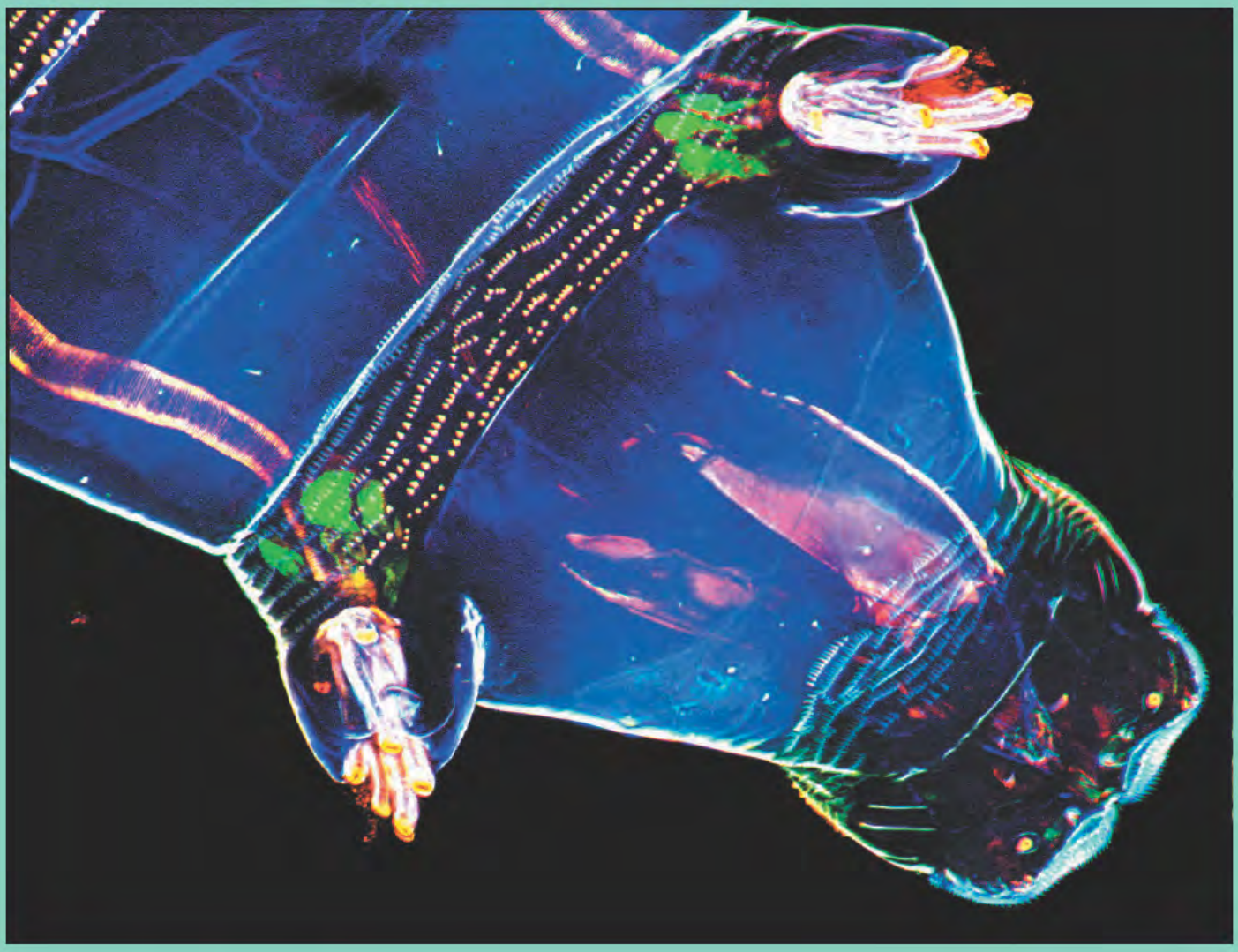
Index of Names

Image of a living two-month sea urchin (*Strongylocentrotus purpuratus*), 100x, dorsal view. Four-pointed juvenile spines and one-pointed definitive spines radiating peripherally, red pigments dotting the surface, and tube feet out of focus at the back (Feng Gao, Eric Davidson lab). See abstract 28.



Legend for the Back Cover Illustration

Confocal reconstruction of the "head" of a *Drosophila* larva. The two large striated orange tubes are the autofluorescent trachea providing air to the larva, opening up in eight telescopic spiracles. Note the large green fluorescent cells at the base of these, revealing expression of Neural Lazarillo in the anterior glands. (Julien Muffat, Seymour Benzer lab). See abstract 227.