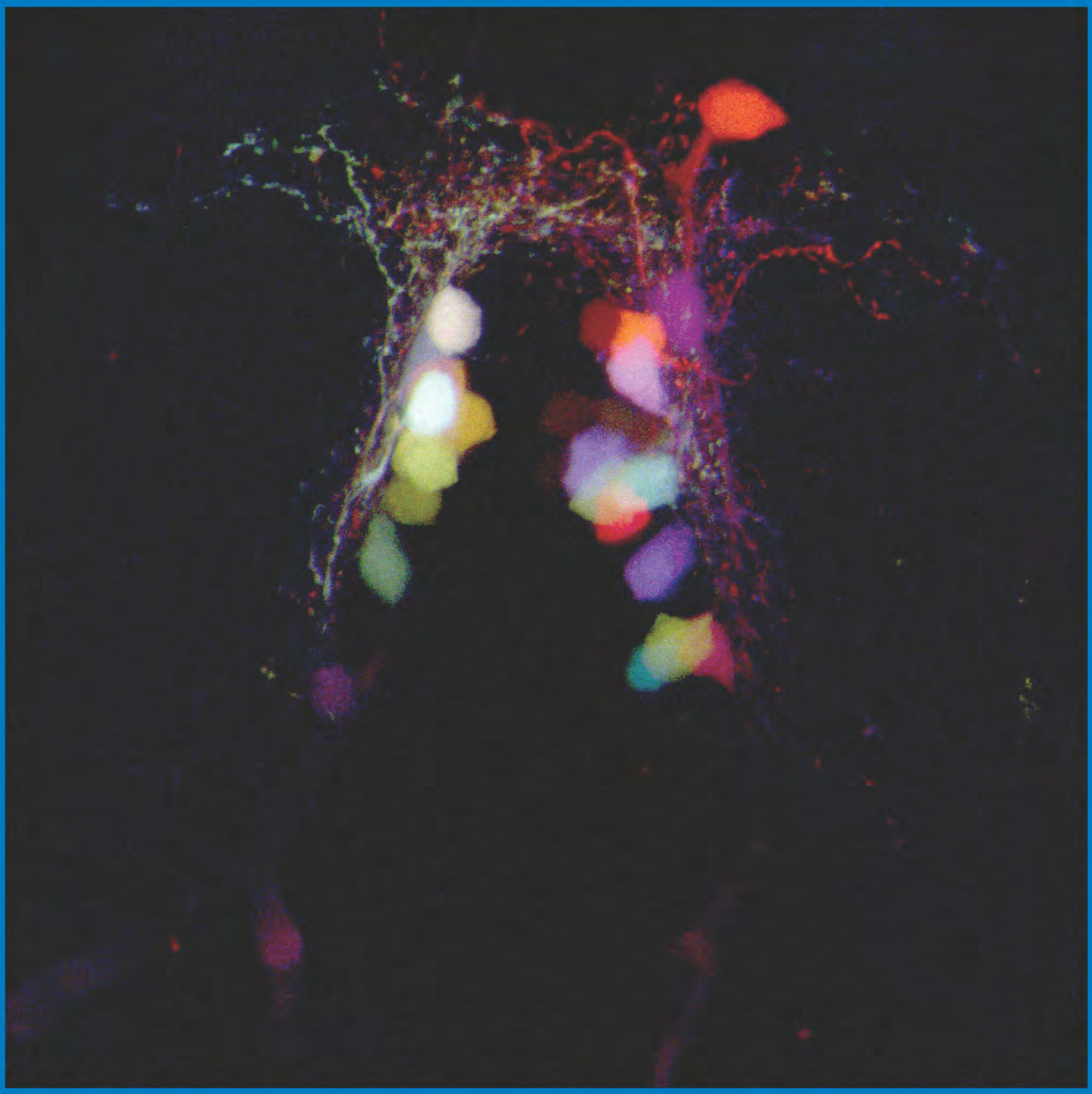

CALTECH Biology Annual Report 2010

Front Cover Illustration

Zebrafish QRFP neurons labeled using Brainbow.

This image shows neurons in the larval zebrafish hypothalamus that express the neuropeptide QRFP. Each neuron is labeled in a different color using the Brainbow technique, a useful approach to visualize the axonal and dendritic projections of individual neurons within a genetically defined population of neurons. The optical transparency and relatively simple neural circuits of zebrafish larvae makes them well suited for this approach.

- David Prober's laboratory

Back Cover Illustration

See Abstract 147

The virtual worm: 3D renderings of *Caenorhabditis elegans, Christian Grove*

The organization of biological data into concise, intuitive, and engaging modes of communication is becoming increasingly important. This model is to-scale three-dimensional rendering of an adult hermaphrodite, representing all the major body tissue types including the skin, muscles, intestine, germline, and the nervous system at the resolution of individual cells. This model is being used to summarize gene expression data in WormBase, the major public database of nematode biology and genetics.

- Christopher Grove, Paul Sternberg's laboratory

Division of Biology

California Institute of Technology

Pasadena, California

Annual Report
2009 - 2010

BIOLOGY

Yolanda Duron, Annual Report Coordinator

Research Reports

Biological research summarized in this report covers the time period from June, 2009 through July, 2010. 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.

TABLE OF CONTENTS

INTRODUCTION

DEVELOPMENTAL AND REGULATORY BIOLOGY

BRUCE A. HAY, PH.D.

SARKIS MAZMANIAN, PH.D.

ELLIOT M. MEYEROWITZ, PH.D.

ELLEN V. ROTHENBERG, PH.D.

STRUCTURAL, MOLECULAR AND CELL BIOLOGY

MOLECULAR, CELLULAR, AND INTEGRATIVE NEUROSCIENCE

SENIOR RESEARCH FELLOWS WITH INDEPENDENT LABS

BROAD SENIOR RESEARCH FELLOWS:

STIJN CASSENAER, PH.D.
SOTIRIS MASMANIDIS, PH.D.
ANDREW STEELE, PH.D.
DANIEL WAGENAAR, PH.D.
GUANGYING WU, PH.D.

FACILITIES

INTRODUCTION

Assistant Professor Alexei Aravin joined Caltech's Biology faculty in January 2010 to continue with his research in molecular genetics of small non-coding RNAs. Alexei was awarded his Ph.D. from Moscow State University, Russia. Both he and Dr. Vladimir Gvozdev discovered that small RNAs are involved in the normal development of the fruit fly. During his previous years as a postdoctoral fellow, first at Rockefeller University with Thomas Tuschl, and then at Cold Spring Harbor Lab with Greg Hannon, Alexei continued his work on small RNA pathways, and as a result, extended his studies to mammals. He has developed a biochemical approach in which he purifies small RNA complexes that are specifically expressed in germ cells. As a result this work, a new class of small non-coding RNAs, Piwi-interacting RNA was discovered. This work has opened up a new direction in the fields of RNAi and germ cell development. Alexei found that piRNA provides germ cells with a programmable "cellular immune system" against genomic intruders capable of both, long-term genetic memory of the transposon invasion, and the acute response to active transposons. This work has identified the functions of many genomic regions that were previously uncharacterized while explaining the molecular mechanism of the previously mysterious genetic loci that serves as the master control loci for transposon silencing. Alexei's goal is to understand the biogenesis of small non-coding RNAs and their functions in development. He has recently moved to a newly renovated lab on the second floor of the Braun building.

LAWRENCE L. AND AUDREY W. FERGUSON PRIZE, 2010

Bronner **Betancur** **Deshaies**

Dr. Paola Betancur is the winner of the Ferguson Award for the 2009-2010 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. Betancur performed her graduate studies in the laboratory of Professor Marianne Bronner.

Her thesis focused on the identification and molecular dissection of the regulatory region of a key transcription factor, Sox10, which is important for specification and differentiation of neural crest cells. She identified the first early neural crest enhancer and found that is was directly activated by the combinatorial input of three transcription factors, Sox9, Ets1 and cMyb. Her work has important implications for understanding and expanding gene regulatory interactions responsible for formation of the neural crest, a cell population that forms critical parts of the nervous system and craniofacial skeleton of vertebrate embryos.

PROFESSORIAL AWARDS, 2009 - 2010

Richard A. Andersen, James G. Boswell Professor of Neuroscience, gave the Herman P. Schwan Distinguished Lecture at the University of Pennsylvania, 2009

David J. Anderson, Seymour Benzer Professor of Biology, received the Paul G. Allen Foundation Distinguished Investigator Award.

Alexei Aravin, Assistant Professor of Biology, received the Ellison Medical Foundation New Scholar in Aging Award; also, received the 2010 NIH Director's New Innovator Award.

David Baltimore, Robert Andrews Millikan Professor of Biology, Nobel Laureate, President Emeritus, was honored as follows: (2009) Builders of Science Award, Research America, and The John P. McGovern Award in Science; (2010) Gregor Mendel Award; and, was on the President's Council of Advisors on Science and Technology (PCAST) Working Group on Influenza Vaccinology.

Pamela Bjorkman, Max Delbrück Professor of Biology, was awarded the 2010 NIH Director's Pioneer Award.

Marianne Bronner, Albert Billings Ruddock Professor of Biology, was honored as follows: (2009) Elected Fellow, American Academy of Arts and Science; President, Society for Development Biology; NIDCR Council. (2010) Secretary, International Society for Developmental Biology; and also, Vice-Chair, Board of Directors, Gordon Research Conference.

Raymond Deshaies, Professor of Biology, Executive Officer, was selected to give the second annual Anniversary Lecture for the Scottish Institute for Cell Signalling (SCILLS), Dundee, Scotland; and, was also selected to give the Max Birnstiel Lecture, Institute of Molecular Pathology, Vienna, Austria.

Scott E. Fraser, Anna L. Rosen Professor of Biology, was awarded the Distinguished Researcher in Bioengineering, UC Riverside.

Grant J. Jensen, **Professor of Biology,** was appointed to the Defense Sciences Study Group of the Institute for Defense Analysis, and will serve on the Scientific Advisory Board of the Boulder Laboratory for 3-D Electron Microscopy of Cells, a National Center for Research Resources.

Henry A. Lester, **Bren Professor Biology,** was the keynote speaker at Texas Tech Research Week and also at Vanderbilt Student Research (2010).

Elliot M. Meyerowitz, George W. Beadle Professor of Biology, won, with his coauthors of the paper Hamant *et al*. (2008) *Science* **322**, 1650, the "Prix La Recherche" from La Recherche magazine and the French Ministry of Higher Education and Research. He was also named a Fellow of the American Society of Plant Biologists. He also gave the Storer Lecturers at the University of California, Davis, on March 11 and 12, 2010. He also gave the keynote address at the International Congress of *Arabidopsis* Research in Yokohama, Japan, on June 6, 2011.

Dianne Newman, Professor of Biology and Geobiology, was honored as follows: (2009) Chair, Division K (Physiology and Metabolism), American Society for Microbiology; (2009-present) Scientific Advisory Board, Biosigma, Chile; Scientific Advisory Board, Mascoma Corporation; (2010) Counselor, Division K (Physiology and Metabolism), American Society for Microbiology; and Krumbeing Lecturer, University of Chicago/Northwestern.

Anne P. and Benjamin F. Biaggini Professor of Biological Sciences, Paul H. Patterson, was honored as follows: (2009) Keynote James Cuozzo Memorial Lecturer, Center for Research on Reproduction and Women's Health, University of Pennsylvania; Psychiatry Grand Rounds, University of Colorado Medical Center; (2010) Grand Rounds, Division of Child and Adolescent Psychiatry, Columbia University/New York State Psychiatric Institute; and, Keynote speaker, Boston Conte Center for Schizophrenia Retreat.

David Prober, Assistant Professor of Biology, was awarded the following awards: the Edward Mallinckrodt Jr., Foundation Award, and the Rita Allen Foundation Milton Cassel Scholar.

Paul W. Sternberg, Thomas Hunt Morgan Professor of Biology, was elected vice president and president-elect of the Genetics Society of America.

Doris Y. Tsao, Assistant Professor of Biology, received the following awards in (2009) Alfred Sloan Scholar, NARSAD Young Investigator, John Merck Scholar, Searle Scholar, and became a Klingenstein Fellow; (2010) Presidential Early Career Award for Scientists and Engineers.

Howard and Gwen Laurie Smits Professor of Cell Biology, Alexander J. Varshavsky, received the 2010 Vilcek Prize in Biomedical Research, by the Vilcek Foundation. He also presented the President's Lecture at the Sanford/Burnham Medical Research Institute, La Jolla, CA. He received the Distinguished Lecture in Signal Transduction at the University of California, Irvine.

Other Awards:

Rochelle A. Diamond, Member of the Professional Staff, was awarded the Dr. Fred Shair Award from the Caltech Center for Diversity for work that supported the campus' diversity and inclusion efforts with emphasis on programming for the LGBTQ campus community. The award was presented at the Center for Diversity's Celebration of Excellence, May 19, 2010.

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The Division of Biology hosted the following lectures:

January 21, 2010
Horowitz Lecture

Michael J. Donoghue
G. Evelyn Hutchinson, Professor of Ecology and Evolutionary Biology, Yale University
"Charles Darwin, the tree of life, and the future of biodiversity"

April 23, 2010
Kroc Lecture

Gary Nabel
Director, Vaccine Research Center,
National Institute of Allergy and Max-Planck Institute of Neurobiology, Germany
"Gene delivery, structural biology, and its applications to medicine"

May 21, 2010
Weigle Lecture

Wolfgang Baumeister
Max-Planck Institute of Biochemistry, Germany
"Electron cryo-microscopy: from molecules to systems"
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Pictures from the Ray Owen 95th Birthday Celebration, held November 4, 2010. Photos taken by Bob Paz

Pictures from the Holiday Celebration Down Under, held December 15, 2010. Photos taken by Bob Paz

BIOLOGY DIVISION STAFF

INSTRUCTION AND RESEARCH

ADMINISTRATION

Division of Biology

Stephen L. Mayo, *Chair*
Raymond Deshaies, *Executive Officer for Molecular Biology*
Christof Koch, *Executive Officer for Neurobiology*

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John N. Abelson, Ph.D.,
George Beadle Professor of Biology

Charles J. Brokaw, Ph.D.,
Biology

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

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

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

Melvin I. Simon, Ph.D.,
Anne P. and Benjamin F. Biaggini Professor of Biological Sciences

James H. Strauss, Ph.D.,
Ethel Wilson Bowles and Robert Bowles Professor of Biology

Senior Research Associate Emeriti

Roy J. Britten, Ph.D.,
Distinguished Carnegie Senior Research Associate in Biology

Anne Chomyn, Ph.D.
Ellen G. Strauss, Ph.D.

Professors

Ralph Adolphs, Ph.D.
Bren Professor of Psychology and Neuroscience;
Professor of Biology

John M. Allman, Ph.D.
Frank P. Hixon Professor of Neurobiology

Richard A. Andersen, Ph.D.
James G. Boswell Professor of Neuroscience

David J. Anderson, Ph.D.*
Seymour Benzer Professor of Biology;
Investigator, Howard Hughes Medical Institute

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

Pamela Bjorkman, Ph.D.*
Max Delbrück Professor of Biology;
Investigator, Howard Hughes Medical Institute

Marianne Bronner-Fraser, Ph.D.
Albert Billings Ruddock Professor of Biology

Judith L. Campbell, Ph.D.
Professor of Chemistry and Biology

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Eric H. Davidson, Ph.D.
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** Joint appointment with Howard Hughes Medical Institute*

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** Joint appointment with Howard Hughes Medical Institute*

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2009- 2010

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Alexandria Berry[2]
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Ronald Bryan[1]
Anna Basalova Buchman - B.S., M.S
Charles Bugg[2]

Gil Carvalho - M.D.
Kuang-Jung Chang - B.S., M.S.
Aadel Chaudhuri - B.S.
Shijia Chen - B.S.
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Cindy Chiu - B.A.
Julie Cho - B.A.
Andrea Choe - B.S.
Janet Chow - B.S.
Suk-Hen Elly Chow - B.S., M.S.

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Sagar Damle - B.S.
John Delacruz[1]
Marissa Morales-Del Real - B.A.
John Delacruz[1]
Emzo de los Santos[3]
Gilberto De Salvo - B.S.
William Dempsey[3]
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Alana Dixson - B.A., M.S., MPH
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Kelly Dusinberre[2]

Eric Erkenbrack - B.A., B.S.

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Xiaodi Hou[1]
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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.

Our laboratory concentrates on studying the cellular and molecular 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. Histone demethylase JmjD2A regulates neural crest specification

Pablo Strobl, Tatjana Sauka-Spengler, Marianne Bronner

The neural crest is a multipotent stem cell-like population that is induced during gastrulation, but only acquires its characteristic morphology, migratory ability, and gene expression profile after neurulation. This raises the intriguing possibility that precursors are actively maintained by epigenetic influences in a stem cell-like state. Accordingly, we report that dynamic histone modifications are critical for proper temporal control of neural crest gene expression *in vivo*. The histone demethylase, JumonjiD2A (JmjD2A/KDM4A), is expressed in the forming neural folds. Loss of JmjD2A function causes dramatic downregulation of neural crest specifier genes analyzed by multiplex NanoString and in situ hybridization. Importantly, *in vivo* chromatin immunoprecipitation reveals direct stage-specific interactions of JmjD2A with regulatory regions of neural crest genes, and associated temporal modifications in methylation states of lysine residues directly affected by JmjD2A activity. Our findings show that chromatin modifications directly control neural crest genes in vertebrate embryos via modulating histone methylation.

2. A key regulatory enhancer for cranial neural crest: Genomic code for Sox10

Paola Betancur, Tatjana Sauka-Spengler, Marianne Bronner

The neural crest is a multipotent, stem cell-like population that migrates extensively in the embryo and forms a wide array of derivatives ranging from neurons to melanocytes and cartilage. Analyses of the gene regulatory network (GRN) driving neural crest development revealed Sox10 as one of the earliest neural crest specifying genes, cell-autonomously driving delamination and directly regulating numerous downstream effectors and differentiation gene batteries. In search of direct inputs to the neural crest specifier module, we dissected the chick Sox10 genomic region and isolated two downstream regulatory regions with distinct spatiotemporal activity. A novel element, Sox10E2 represents the earliest known neural crest *cis*-regulatory element, critical for initiating Sox10 expression in newly formed cranial, but not vagal and trunk neural crest. A second element, Sox10E1, homologous to a previously identified mouse enhancer, acts in later migrating vagal and trunk crest cells. Deep characterization of Sox10E2 reveals Sox9, Ets1 and cMyb as direct inputs mediating enhancer activity. Chromatin immunoprecipitation and gel shift assays demonstrate their direct binding to the Sox10E2 enhancer *in vivo*, whereas mutation of their corresponding binding sites, or inactivation of the three upstream regulators, abolishes both reporter and endogenous Sox10 expression. Using *cis*-regulatory

analysis as a tool, our study makes critical connections within the neural crest GRN, thus establishing the first direct link of upstream effectors to a key neural crest specifier at the cranial level.

3. A Sox10 enhancer element common to the otic placode and neural crest is activated by tissue specific paralogues

Paola Betancur, Tatjana Sauka-Spengler, Marianne Bronner

The otic placode, a specialized region of ectoderm, gives rise to components of the inner ear and shares many characteristics with the neural crest. For example, transcription factors of the SoxE family are expressed in both populations in all vertebrates examined, including the basal lamprey. In avian embryos, the enhancer Sox10E2 has regulatory activity in both the otic placode and cranial neural crest. Here, we show that activity of Sox10E2 in the ear is mediated by three transcription factors, cMyb, Sox8 and Pea3, all of which are necessary for initial expression of *Sox10* in the otic placode, forming the ear. Mutating each of the corresponding binding motifs within Sox10E2 greatly reduces its activity in the otic region. Moreover, simultaneous knock-down of all three factors eliminates not only enhancer expression but also initial Sox10 expression in the ear. These data uncover a new function for cMyb, Sox8 and Pea3 in ear development via a common otic and neural crest enhancer. Interestingly, a different combination of paralogous transcription factors activate this same enhancer in cranial crest, suggesting an evolutionarily conserved mechanism for dual regulation of Sox10 in these tissues of distinct embryological origin.

4. Regeneration of the early cardiac neural crest in chick

Akouavi M. Ezin, Angela Zah, John W. Sechrist, Scott E. Fraser, Marianne Bronner

The cardiac neural crest is a regionally-defined subpopulation of the neural crest (from caudal rhombomere 5 to somite 4) that contributes to the septation of the cardiac outflow tract and formation of aortic arches. Removal of the cardiac neural crest from the chicken embryo after neural tube closure results in an absence of cardiac neural crest derivatives, resulting in severe septation defects, reminiscent of human birth defects. Because of the regeneration of the neural crest seen in some axial levels, we asked whether the cardiac neural crest lacks this ability altogether, or has a regenerative capacity that diminishes with time. We used our previously established dynamic fate map of the chicken neural crest to ablate the region of the neural folds/neural tube that would normally give rise to the cardiac neural crest, and find that ablations at stage 7 do not result in an absence of cardiac neural crest when assayed at stage 13. The regenerated population derives largely from the rostral cardiac crest level including rhombomere 5 and neuroepithelium ventral to the ablation. This regeneration is not at the expense of other neural crest derivatives such as the enteric nervous system. We find that this regenerative capacity is lost by stage 9, consistent with previous reports, suggesting that the there is a relatively narrow temporal window before regeneration becomes restricted.

5. Altering Glypican-1 levels modulates canonical Wnt signaling during trigeminal placode development

Celia Shiau, Na Hu, Marianne Bronner

Glypicans are conserved cell surface heparan sulfate proteoglycans expressed in a spatiotemporally regulated manner in many developing tissues including the nervous system. Here, we show that Glypican-1 (GPC1) is expressed by trigeminal placode cells as they ingress and contribute to trigeminal sensory neurons in the chick embryo. Either expression of full-length or truncated GPC1 *in vivo* causes defects in trigeminal gangliogenesis in a manner that requires heparan sulfate side chains. This leads to either abnormal placodal differentiation or organization, respectively, with near complete loss of the ophthalmic (OpV) trigeminal ganglion in the most severe cases after over-expression of full-length GPC1. Interestingly, modulating GPC1 alters levels of endogenous Wnt signaling activity in the forming trigeminal ganglion, as indicated by Wnt-reporter expression. Accordingly, GPC1 over-expression phenocopies Wnt inhibition in causing loss of OpV placodal neurons. Furthermore, increased Wnt activity rescues the effects of GPC1 over-expression. Taken together, these results suggest that appropriate levels of GPC1 are essential for proper regulation of canonical Wnt signaling during differentiation and organization of trigeminal placodal cells into ganglia.

6. Spatiotemporal control of RNA interference using enhancer driven short-hairpin miR vectors

Katherine Fishwick, Natalya Nikitina, Joanne Tan-Cabugao, Marianne Bronner, Tatjana Sauka-Spengler

RNA interference (RNAi) offers an effective method for specific targeted gene knockdown in many organisms. However, its use in the developing amniote embryo has been problematic due to non-specific, off-target effects. This results from introduction via highly expressing constitutive promoters or multiple cassette designs, yielding high number of exogenous microRNA arms containing embedded small interfering RNAs. Here, we present a vector system controlled by tissue-specific enhancer elements that regulate production of knockdown transcripts at endogenous levels. This system combines effective conditional knockdown with a built in EGFP tag and the flexibility afforded by modular enhancers and short hairpin elements. Importantly, this tool makes it possible to achieve *in vivo* loss-of-function at very early developmental stages with rigorously assessed absence of non-specific effects.

7. Pax2 and Pea3 synergize to activate a novel regulatory enhancer for spalt4 in the developing ear

Meyer Barembaum, Marianne Bronner

The transcription factor ***spalt4*** is a key early-response gene in otic placode induction. Here, we characterize the *cis*-regulatory regions of ***spalt4*** responsible for activation of its expression in the developing otic placode and report the isolation of a novel core enhancer. Identification and mutational analysis of putative transcription factor binding sites reveal that ***Pea3***, a downstream effector of FGF signaling, and ***Pax2*** directly activate ***spalt4*** during ear development. Morpholino-mediated knock-down of each factor reduces or eliminates reporter expression. In contrast, combined over-expression of ***Pea3*** and ***Pax2*** drives ectopic reporter expression, suggesting that they function synergistically. These studies expand the gene regulatory network underlying early otic development by identifying direct inputs that mediate ***spalt4*** expression.

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Summary: The major focus of research in our laboratory is the systems biology of the 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 molecular biology technologies for high throughput perturbation of gene expression in the embryo; a novel high throughput method of *cis*-regulatory analysis; 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); a Nanostring codeset for >180 interesting regulatory genes and some signaling ligands and receptors; and >100 custom recombineered BACs. There is also a rich collection of arrayed cDNA and BAC libraries for many other species of sea urchin, at various degrees of relatedness to this one. The genome of *S. purpuratus* has been sequenced at HGSC (Baylor) and annotated. We utilize additional experimental echinoderm models for evolutionary GRN comparisons, viz. the starfish *Patiria miniata* also of local provenance, and the pleisiomorphic "pencil urchin" *Eucidaris tribuloides*. The embryos of both these animals prove to be as excellent subjects 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 causal explanations of major developmental phenomena directly emerge, is the GRN system level. The main research initiatives in our laboratories at the present time are as follows:

(i) Analysis of the gene regulatory network underlying endomesoderm specification in *S. purpuratus* embryos: Many of the individual projects reported below are contributing to understanding of this GRN. At present, over 60 regulatory and signaling genes have been linked into this network. The architecture of the network is emerging from an interdisciplinary approach in which high resolution spatial and temporal regulatory gene expression data are combined with perturbation data obtained by gene expression knockouts and other methods to provide a causal explanation of the observed embryology. A predictive model of the GRN is emerging 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. In current work, as described below, we have discovered the dynamic mechanism by which the endoderm/mesoderm fate decision is made, as well as that by which future anterior vs. posterior endoderm is specified; and are in process of clarifying and expanding the oral and aboral mesodermal GRNs, which produce different mesodermal cell types.

ii. Oral and aboral ectoderm GRNs: In an effort to extend GRN analysis to much of the embryo, we have recently attained draft GRNs for oral and aboral ectoderm specification including about 30 more regulatory genes (the remaining major territory, the apical neurogenic region, is being studied in other sea urchin laboratories). The ectoderm is a complex mosaic of spatial regulatory states. The aboral ectoderm generates a single cell type, but the region abutting the endoderm expresses specific regulatory genes, as also on the oral side, and the oral ectoderm also produces the mouth, columnar "facial" epithelium and the ectodermal signaling stripes which determine the location of the skeletal rods. Separating oral and aboral domains and oral and apical domains is the neurogenic ciliary band, which expresses its own regulatory state. Several current projects are focused on the GRNs underlying all these regional specifications.

iii. High throughput cis-regulatory analysis, and its impact on GRN analysis: A recent technological breakthrough is revolutionizing the processes of GRN validation and discovery, as well as vastly improving the efficiency with which *cis*-regulatory control systems can be analyzed. This is the development of multiplexed *cis*-regulatory analysis using vectors marked with "bar-coded" sequence tags, up to 130 of which can be injected together into a single batch of sea urchin eggs. The individual vectors are regulated independently *in vivo* and their outputs can be deconvolved at once by QPCR or NanoString technology. Expression of individual vectors can also be examined spatially since each vector also expresses GFP. GRN structure is validated by *cis*-regulatory confirmation of the predicted linkages, and this can now be done on a large scale. Overall, *cis*-regulatory validation is converting the GRN from a model proposition into a hardwired map of the genomic control logic for this portion of development. Even more significantly the effects of perturbations of gene expression can now be determined at the same time on endogenous genes and on their *cis*-regulatory systems isolated by high throughput functional genomic scans. This transforms the process of GRN discovery.

iv. Specific cis-regulatory projects using high throughput methods: *Cis*-regulatory systems at certain GRN nodes are of particular importance, and many of these are the subjects of particular experimental analysis. During this year *cis*-regulatory systems of the following genes, among others, were studied at the level of their sequence specific inputs and their functional meanings (some of these projects are now complete and have been or will soon be published): *alx1, tgif, hex, foxa, brachyury, gcm, hnf6, irxa, tbx2/3, foxg, gsc.*

v. Embryonic transcriptome database and analysis: We have embarked on a large-scale transcriptome sequencing effort. RNAs from many timed embryonic stages, from feeding larval stages, and from all accessible adult tissues is being sequenced. Three valuable kinds of data are obtained and after computational analysis are being mounted on our public sea urchin genomics database: (1) the sets of transcripts expressed in each stage and tissue; (2) the prevalence of each and their dynamic changes during development; and (3) what transcripts of given ontological classes of interest are present in each transcriptome, such as transcripts coding for immune proteins, for cytoskeletal proteins, for transcription factors, for signaling factors, for biomineralization proteins, etc. The ontological classes were based on the annotations of genes in the *S. purpuratus* genome project.

vi. Physical isolation of embryonic cells expressing given regulatory states: Another technological breakthrough has been the development of methods for disaggregation of sea urchin embryos to the single cell level, and efficient FACS sorting. The cells are sorted on the basis of expression of recombineered BAC vectors, in which a flourophore is expressed under control of the *cis*-regulatory system of a gene canonically representing a given domain-specific regulatory state. Recoveries of expressing cells are quite acceptable. The availability of this technology leads in two different directions: First, it allows us to characterize the spatial expression of large sets of isolated *cis*-regulatory modules isolated by high throughput genomic regulatory scans, as above. Thus, by detecting the bar code vector tags, we can determine which of the *cis*-regulatory modules are expressed in cells also expressing each of the regulatory states of interest. Second, we can obtain the transcriptomes of cells expressing given regulatory states. This in turn will lead to construction of "Global GRNs" in which the control systems of all specifically expressed downstream genes (of given ontological classes) are discovered and linked into our current upstream GRNs.

vii. Evolutionary co-option at the *cis*-regulatory level: The major mechanism of evolutionary change in GRN structure is co-option of regulatory and signaling genes to expression in new spatial/temporal domains of the developing organism. This means change of *cis*-regulatory modules at the sequence level, so that they respond to different regulatory states. An excellent example is the use of Delta-Notch signaling to promote mesoderm specification in sea urchins, but to promote endoderm specification in sea stars (the sea urchin mode is the derived co-option). Sea stars and sea urchins shared a last common ancestor about 500 million years ago. To determine what happened in the lineage leading to sea urchins, we are carrying out a *cis*-regulatory study of sea

star *delta*, for comparison to sea urchin *delta*, including cross-specific transfer of expression constructs.

viii. *Eucidaris tribuloides*, an evolutionary window on the origins of the euechinoid endomesoderm specification GRN: The euechinoids are the modern sea urchins, of which the main research model is *Strongylocentrotus purpuratus*, for the last 40 years our laboratory workhorse. The euechinoids diverged from the Paleozoic precursor echinoid lineage about 275 million years ago. *Eucidaris tribuloides* is a descendant of the other surviving branch of echinoids descendant from the same common ancestor stock. Its endomesodermal specification process is quite different from that of *S. purpuratus*; for example, it lacks a skeletogenic micromere lineage and therefore the signaling inputs provided by that lineage. We are beginning to characterize the pleisiomorphic specification GRN of *E. tribuloides* to determine exactly how that of *S. purpuratus* evolved since divergence. In addition, we are attempting to reprogram the development of the skeletogenic cell lineage in *E. tribuloides*, by inserting regulatory apparatus from *S. purpuratus*. We term this Synthetic Experimental Evolution. We have already been successful using this approach in causing the skeletogenic lineage of *E. tribuloides* to invaginate precociously, just as does that of *S. purpuratus.*

ix. New genomics projects: A large amount of additional echinoderm sequence is in process of being obtained. The leaders in this project are Richard Gibbs and Kim Worley at the Baylor College of Medicine Human Genome Sequencing Center (BCM-HGSC) in Houston, in close collaboration with us. The initial target is the genome of *Lytechinus variegatus*, to be followed by the genome of the sea star referred to above, *Patiria miniata*, and that of *E. tribuloides*. Much additional genome sequence of *S. purpuratus* is also being obtained, so as to significantly improve its quality; and earlier skim sequences of two congeners, *S. franciscanus* and *Allocentrotus (Strongylocentrotus) fragilis* are also being augmented. All of these data are being curated and mounted on the public genome databases that we maintain and continuously augment.

x. Progress in theory, including GRN structure/function relations; BioTapestry computational platform; GRN dynamics: Several apparent principles of GRN organization are emerging. (a) GRNs are composed of modular subcircuits executing discrete logic functions; (b) These subcircuits evolve at different rates within the same GRN and may have diverse evolutionary origins; (c) Multiple, overlayered subcircuits are brought to bear on given developmental processes, including dynamic lockdowns by feedback circuitry, to ensure that they function accurately and reproducibly: the "wiring" is clearly not parsimonious in design; (d) The "over-wired" design accounts for the resilience of the developmental process, including the regulative ability of the embryo, and also its resistance to experimental rewiring except at the periphery of the GRN (top or bottom); and (e) Different processes, e.g., embryonic spatial specification, terminal differentiation, physiological response, are controlled by differently structured GRNs, which have different depths and are composed of different types of subcircuit. The GRN visualization software BioTapestry, developed by our collaborator Wm. Longabaugh (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. In addition, together with my collaborator Doug Erwin (Smithsonian Institution) two articles on evolution which dealt with new concepts were published, one on the Precambrian origins of developmental GRNs; one on *cis*-regulatory cooption theory. We also published a review on mathematical treatments of RNA synthesis dynamics, transcriptional processes, and some GRN subcircuit dynamics.

The Center for Computational Regulatory Genomics
R. Andrew Cameron, Director

The Center for Computational Regulatory Biology and its subsidiary, the Genomics Technology Facility, is an integrated unit whose goal is to develop, refine and test computational approaches in genomics broadly and *cis*-regulatory analysis specifically. It consists of three overlapping areas of activity. The Genomics Facility is a high-throughput library arraying and printing operation that supplies macroarray libraries and clones to the community. The molecular biology branch tests and refines techniques associated with molecular developmental biology, gene transfer, and the routine aspects of *cis*-regulatory analysis developed in the Davidson laboratory. The computational branch is an enterprise that supplies software to the sea urchin developmental biologist and maintains databases fundamental to the Sea Urchin Genome Project, an initiative that began in the Davidson laboratory and at the Genomics Technology Facility.

Genomics Technology Facility
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 three 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. 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. Each year, we print 80-90 new filters for these as needed. In addition,

we make and array new libraries to support genomics efforts in echinoderms and other lower deuterostomes. Research materials have been provided to research laboratories ranging from University of Washington to Stazione Zoologica Anton Dohrn (Italy).

Our BAC libraries have provided the source material for *in vitro* recombineered BACs used by the whole research community. The recombineering project has already produced >100 different recombinant BACs from five echinoderm species for use as reporter constructs. This includes BAC reporter constructs in which a fluorescent protein coding region (GFP, RFP, mCherry) has been inserted into the coding region of a gene of interest as well as numerous constructs in which *cis*-regulatory modules (CRM) have been deleted or mutated. Requests for recombineered BAC clones come from the research community at large, as well as out laboratory. We expect that as more echinoderm genomes are made available the demand will be stimulated yet again. All of the recombineered BAC clones once tested are listed on the SpBase website and made available to the community.

Our collaboration with the Baylor College of Medicine, Human Genome Sequencing Center continues on several fronts. We provide material for, and later computational analysis of a series of new next-generation genome sequencing projects for *Lytechinus variegatus*, *Patiria miniata* and *Eucidaris tribuloides*. It is anticipated that a combination of Roche 454 and Illumina read pools will be used to assemble genomes for these species. In addition, mRNA samples from various development stages are being pooled and sequenced to support the genome efforts.

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://spbase.org/). 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.

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 entire genome sequences on the cluster in various ways as part of the genome annotation effort. We further expect to queue search jobs from the genome database through this cluster.

8. Regulatory logic of endoderm development in pre-gastrula stage sea urchin embryos

Isabelle Peter, Jina Yun

Based on genome-wide transcription factor gene expression analyses, we currently know of fourteen regulatory genes expressed in endodermal precursor cells in pre-gastrula stage sea urchin embryos. Two embryonic cell lineages, deriving from veg2 and veg1 cells, contribute to the formation of the gut and we have analyzed the spatial expression of regulatory genes in respect to these two domains. To determine the regulatory interactions between these regulatory genes, we have perturbed the expression of each transcription factor by injection of gene-specific morpholinos and analyzed the changes in the expression levels of all other regulatory genes. We found that endodermal regulatory gene expression does not occur uniformly in these endodermal precursor cells. At early blastula stages, a few hour after the formation of the two cell lineages, there are nine regulatory genes expressed, all of them restricted to either one of the endodermal domains. Eight of these regulatory genes are expressed in the veg2 cell lineage, most of them under the transcriptional control of Tcf/β-catenin, whereas only one regulatory gene is expressed specifically in the veg1 lineage. In the veg2 lineage, Hox11/13b activates the expression of *blimp1b*, *foxa* and *brachyury*. These four genes are also controlled by Tcf/β-catenin and expressed in the entire veg2 cell lineage at early blastula stage. After 18 h of development, these genes become repressed in the part of veg2 derived cells which give rise to mesodermal cell types and this repression occurs downstream of Delta/Notch signaling, which is a driver of mesodermal specification. After this stage, the expression of these regulatory genes is therefore restricted to endodermal precursor cells. The transcriptional control of the veg1-specific gene *eve* involves activation by Tcf/β-catenin, auto-repression and repression by Hox11/13b, which is present in veg2 derived cells. At mesenchyme blastula stage, there are three

additional regulatory genes expressed in the veg1 lineage: one under the control of Eve (*hoβx11/13b*), one under the control of Hox11/13b (*brachyury*), and one under the control of Eve and Brachyury (*hnf1*), which results in a feed-forward circuitry. The transition of *hox11/13b* expression from veg2 cell lineage to the veg1 lineage involves auto-repression like has been observed for *eve*. Within the veg2 lineage, there are meanwhile nine regulatory genes expressed, two of which responsive to the perturbation of Eve expression, which predicts a signal connecting the two regulatory networks which are active in veg1 and veg2 endodermal cells. Taken together, these regulatory interactions explain the formation of distinct regulatory states in the two endodermal cell lineages and propose a mechanism by which early embryonic geometry is translated to the anterior/posterior polarity of the larval gut.

9. *Cis*-regulatory analysis of ectodermal genes

Enhu Li

Cis-regulatory modules integrate inputs of transcriptional factors, and define the logic of temporal and spatial expression. *Cis*-regulatory analysis of regulatory genes can be used to authenticate the predicted linkages of gene regulatory network inferred from perturbation assays, as well as an independent tool to identify interactions among regulatory genes. In this project we took a systematic approach to identify the *cis*-regulatory modules of key ectodermal genes, including *gsc*, *foxg*, *not*, *nk1*, *lim1*, *sip1*, and *tbx2/3*. These seven ectodermal genes carry essential roles in specify lineages of ectodermal cells.

A tag-based assay was adopted in the *cis*-regulatory analysis, which involves the following steps: dissecting the previous transcriptionally active genomic regions to smaller pieces for mutagenesis study; scanning for additional active modules; testing the response of *cis*-regulatory modules to various MASO; mutagenesis of known transcription factor-binding sites and studying the resultant temporal and spatial expression.

By scanning 60 kb region upstream of *foxg* gene, we identified four modules driving *foxg* expression, including a proximate module immediately upstream of *foxg*, and three distal modules 36 to 40 kb upstream. The most distal module is active during gastrular stage, and contributes to the cilliary band expression of *foxg*. 5′ proximate and the middle distal module are active throughout early blastula to late gastrula. A distal repressive module was found which prevents premature expression of *foxg* during early blastular stage. Applying the same approach, we also identified smaller *cis*-regulatory modules (1 kb) inside the previously known transcriptionally active regions (4-5 kb), and they are capable of driving proper expression when endogenous genes are active. Currently, various MASO are being tested, and mutagenesis of predicted binding sites is generated to authenticate the predicted linkages. Information gathered in this project will greatly improve our understanding of the sea urchin ectodermal gene regulatory network.

10. Mesoderm specification in sea urchins

Stefan C. Materna

Reception of a Delta/Notch (D/N) signal originating in the skeletogenic cells is essential for specification of mesodermal cells. The two primary targets genes are *gcm*, and *gatae* that initially are expressed in all future mesodermal cells. Eventually, and concurrent with the ingression of the skeletogenic cells, the ring of mesodermal cells is subdivided into an oral and aboral region. The aboral side gives rise to pigment cells and retains expression of *gcm* and *gatae*, while their expression is lost in the oral mesoderm. Instead, genes specific to oral mesoderm (*prox*, *gatac*, *ese*, and *scl*) are activated. Cells that express these genes will adopt blastocoelar cell fate. Despite the lag between the initial activation of *delta* in the neighboring skeletogenic cells, the expression of oral mesodermal genes is strongly dependent on the D/N signal as revealed by perturbation experiments affecting this signaling pathway.

Concurrent with the subdivision of the mesoderm, the mesodermal cells themselves start to express the Delta ligand. To confirm that it is indeed the early phase of D/N signaling emanating from the skeletogenic cells that activates the expression of oral mesodermal genes, D/N signaling was inhibited just prior to the start of this second phase with a specific inhibitor. As expected, pigment cell and blastocoelar cell genes were not affected by this perturbation and both cell types formed as in untreated embryos. However, a different gene (*foxy*) was downregulated in this experiment. *foxy* is expressed in the cells that will contribute to the coelomic pouches at the tip of the invaginating archenteron. Some mesodermal cells that do not become specified as either pigment or blastocoelar cells remain in this area and contribute to the coelomic pouches as well as the circum-esophageal muscle. Thus, the later D/N signal emanating in the mesoderm is important for the proper specification of these cell types.

The subdivision of the mesoderm proceeds along the oral-aboral axis of the embryo. This axis is entrained by *nodal* expression in the oral ectoderm. This member of the TGF beta family activates oral genes in the ectoderm and is also necessary for the activation of the oral mesodermal genes. H owever, there is a significant lag in between *nodal* activation and the onset of expression of *prox* and *gatac*. Perturbation of *nodal* expression revealed the homeobox gene *not*, among others, to be a downstream target of Nodal signaling. Because *not* is expressed early enough to have accumulated to significant levels by the time *prox* and *gatac* are activated we postulated that this gene may be a missing link between Nodal and oral mesodermal genes. Indeed, knockdown of *not* expression significantly reduces the expression level of oral mesodermal genes. Thus, activation of oral mesodermal genes is combinatorial with inputs from both D/N and Not, a hypothesis that can be confirmed at the *cis*-regulatory level.

11. Information processing at the *foxa* node of the sea urchin endomesoderm specification network

Smadar Ben-Tabou de-Leon

The *foxa* regulatory gene is of central importance for endoderm specification across Bilateria, and this gene lies at an essential node of the well-characterized sea urchin endomesoderm gene regulatory network. Here we experimentally dissect the *cis*-regulatory system that controls the complex pattern of *foxa* expression in these embryos. Four separate *cis*-regulatory modules cooperate to control *foxa* expression in different spatial domains of the endomesoderm, and at different times. A detailed mutational analysis revealed the inputs to each of these *cis*-regulatory modules. The complex and dynamic expression of *foxa* is regulated by a combination of repressors, a permissive switch and multiple activators. A mathematical kinetic model was applied to study the dynamic response of *foxa cis*-regulatory modules to transient inputs. This study shed light on the mesoderm – endoderm fate decision and provides a functional explanation, in terms of the genomic regulatory code, for the spatial and temporal expression of a key developmental control gene.

12. Quantitative analysis of *cis*-regulatory modules and networks

Jongmin Nam

Cis-regulatory components of the genome are crucial players of gene regulatory networks. Efficient discovery and functional characterization of *cis*-regulatory modules (CRMs), and their integration into gene regulatory networks have remained a major challenge in genomic regulatory biology. To accelerate these processes, we recently developed a set of "barcoded" reporters that enable us to track *in vivo* activities of ≥100 CRMs simultaneously. The most recent version of "barcoded" reporters, named Nanotags, is compatible with NanoString RNA counting method further accelerating quantitative discovery and characterization of CRMs. We are in the process of combining Nanotags with FACS for the spatial characterization of CRMs and also with perturbation experiments to efficiently study interactions between *cis* and *trans*-regulatory networks using sea urchin embryos as model systems.

13. A high-throughput *cis*-regulatory analysis of late endoderm development in sea urchins

Jonathan Valencia

Early in sea urchin embryonic development, the endomesoderm territory is subdivided into regions with distinct regulatory states. One of these regions, the endoderm, will soon give rise to the tripartite gut, consisting of fore-, mid-, and hind-gut domains. This dynamic process is mediated by a network of regulatory factors that are functionally connected through their *cis*-regulatory modules (CRMs). Using a novel high-throughput DNA-tag approach (i.e., 129 individual "bar-coded" GFP expression reporters), we aim to extend the current endoderm gene regulatory network by identifying and authenticating new and additional inputs from genes expressed late in endoderm development.

Currently, we are in the process of predicting and identifying active CRMs for 20 genes that operate in the late endoderm. We use phylogenetic footprinting to uncover conserved regions of regulatory DNA, i.e., putative CRMs. This is followed by verification in a gene transfer assay using the DNA-tag approach. Here, the unique "bar-coded" DNA tags for each CRM is measured either by Q-PCR or Nanostring technologies. Once we have temporally characterized the active CRMs, we will focus on their spatial characterization. By employing fluorescence-activated cell sorting after co-injection of a endoderm marker BAC expressing RFP, we will quantitatively compare DNA tag enrichment versus depletion in RFP positive and RFP negative cells. Additionally, we will establish regulatory linkages using perturbations accomplished with morpholino anti-sense oligonucleotides against putative regulatory inputs. Combining these data will allow us to construct a new provisional endoderm network, which will direct future *cis*-regulatory analyses.

14. *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. purp*.) orthlog 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. The regulatory module controlling the late expression from MB to dispersed PC continues as the main focus. 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. Beginning around MB stage, two elements within this module are critical. An auto-regulatory site in the form of a consensus site match to the canonical GCM binding site helps maintain *Spgcm* expression levels. Additionally, *cis*-perturbation analyses have demonstrated an additional short element is even more essential. This element is a 6/8 match to the consensus-binding site of the homeo-domain protein Six1, which has been shown to be a target gene of GCM and is expressed in the same cells at this embryonic stage. Characterization of the element(s) that mediate a dramatic down regulation of *Spgcm* on the oral side of the SMC domain in the early MB continues to be a goal. 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.

15. *Cis*-regulatory analysis of *Strongylocentrotus purpuratus alx1* gene

Sagar Damle

The *SpAlx1* gene encodes a paired class homeodomain transcription factor that is deployed during embryonic skeletogenesis in the sea urchin. Its expression kinetics show two peaks – the first and strongest occurring at midblastula stage (roughly 11 hpf) and the second at mesenchyme blastula stage (24 hpf). It is expressed initially in the large micromeres at early blastula stage (7 hpf) and continues as these cells develop into primary mesenchyme (PMC) and ultimately to skeletogenic cells. In the sea urchin embryo, Alx1 is necessary for ingression of PMCs into the blastocoel, migration, and skeletogenesis through the production of skeletal matrix proteins. A *cis*-regulatory analysis is being performed on the Alx1 gene for the purpose of identifying the direct regulatory inputs and demonstrating how they interact to regulate its expression.

The *cis*-regulatory modules controlling both early and late expression are contained 6 kb upstream of the start site of transcription. GFP reporter constructs containing this region are capable of recapitulating spatially the endogenous Alx1 expression pattern. This sequence contains three conserved *cis*-regulatory modules roughly 500 bp in length. The proximal module contains HesC-binding sites and restrict expression to domains that lack the Hes-c repressor – namely the large micromeres at early blastula stage and primary and secondary mesenchyme prior to hatching blastula stage (16 hpf). The middle and distal modules contain binding sites for amplifiers of expression and are necessary for producing the 11 hour expression peak. Binding site mutation and MASO analysis has identified Ets1 as an early initiator of Alx expression. Using similar approaches, we have shown that the Alx1 protein is itself a direct autoactivator of expression that is critical for establishing 50% of the peak expression. At the same time, Alx1 protein is necessary for downregulating mRNA expression after the first peak, as MASO knockdown of Alx1 expression disrupts autorepression. Systematic deletion analysis of the 6kb reporter construct has been unsuccessful at identifying the sequence necessary for autorepression. However, the mechanism of feedback repression is currently being investigated. One hypothesis is that Alx1 protein homodimerizes when at high concentrations. This homodimerization may disrupt its ability to act as an activator in this context. To test this, we are constructing an obligate homodimer form of Alx1 and overexpressing it in the developing embryo. We will check its effect on endogenous Alx 1 transcription as well as on *cis*-regulatory reporter constructs.

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

Lydia Dennis, R. Andrew Cameron

Previously we have identified a minimal enhancer for *brachyury (Sp-Bra),* a gene intermediate in the endomesoderm specification gene regulatory network. Up to the peak of embryonic expression at 24 hrs, it recapitulates, in time and space, the expression of the endogenous gene. This enhancer contains binding sites for Sp-Tcf, a transcription factor that is activated by the Wnt pathway and Sp-Gatae, the output of the endomesoderm kernel subcircuit. An Otx binding site in the larger intronic sequence that contains the minimal enhancer also plays a role in activating this gene. These experiments have taken advantage of BAC clones recombineered with either GFP or RFP reporters. Our current hypothesis for *brachyury* early transcriptional control is: a wnt activity in veg2 endoderm precursors leads to the nuclearization of beta-catenin and the activation of *Sp-Tcf* in those cells. Thus, *brachyury* transcription elevates beginning at about 10 hours pf. Later as *Sp-Gatae* and *Sp-Otx* begin to be expressed in these cells the expression is pushed to the maximum at 24 hours.

To confirm a recent perturbation study that places upstream of *Sp-Bra,* we have employed a morpholino-antisense oligonucleotide (MASO) that abolishes the translation of the Hox11/13b protein. When co-injected with the SP-Bra GFP BAC, this MASO greatly reduces the expression of GFP indicating that the input from *Sp-Hox11/13b* is likely to be direct. We are no conducting experiments to see if the intron enhancer or its minimal fragment is responsive to *Sp-Hox11/13b.*

17. The control apparatus governing transcription of the *Onecut1* gene during early embryonic development of *Strongylocentrotus purpuratus*

Julius C. Barsi

The biological process that drives early embryonic development forward continues to be systematically addressed in *Strongylocentrotus purpuratus*. The pivotal events capable of explaining the progression from zygote to gastrula have for the most part, been identified with molecular resolution. In the attempt to extend this level of analysis towards later stages of embryogenesis, our efforts are now focused on ectoderm specification. This project in particular, aims to elucidate the mechanism by which the neurogenic ciliated band forms; a structure that will ultimately serve the larva for propulsion. The gene known as *Onecut1*, to our knowledge is the very first transcription factor (TF) to be expressed within this domain. Next generation gene expression analysis combined with an innovative perturbation approach, has already revealed the regulatory gene interactions operating downstream of this TF. The direct inputs governing transcription of *Onecut1* itself however, remain to be identified. This promises to be an exceptionally interesting *cis*-regulatory control apparatus, as it will surely have to integrate a range of intercellular signals in order to produce such a discrete pattern of

expression. Functional dissection of the *Onecut1* locus has thus far revealed several regions of interest. Determining whether a particular reporter construct accurately reflects that of the gene of interest, becomes more challenging as embryonic complexity increases. In order to address this and other potential obstacles such as protracted GFP degradation, all reporter constructs were assessed both by quantitative real-time PCR, as well as double fluorescent whole mount RNA *in situ*. In this way, GFP mRNA levels are assessed in relation to the endogenous expression pattern generated by the gene of interest. This approach has been used to interrogate 55Kb of non-coding sequence and successfully narrowed down the search space thirteen fold. Thus, three fragments of noncoding sequence have been identified; each capable of recapitulating distinct aspects of *Onecut1* expression. Future effort will focus on narrowing down the sequence to the point that individual TF binding sites may be subject to mutational analysis.

18. Developmental transcriptomes

Qiang Tu

To establish an accurate model of the global gene regulatory network governing embryonic development, it is critical to measure gene activity in a systematical manner. The emerging high-throughput sequencing technologies now make it possible to survey the whole transcriptome rapidly and precisely. We have commenced to profile gene expression throughout development of the sea urchin, specifically encompassing embryogenesis all the way through metamorphsis, as well as assessing a variety of adult tissues.

Samples were collected from numerous developmental stages relevant to our studies, from which total RNA was then extracted via guanidinium thiocyanate phenol/chloroform (TRIzol), mRNA purified by dT beads (Dynal), and fragmented by base hydrolysis such that it could be used as a template for generating cDNA (Superscript). New generation sequencing technology requires additional manipulation. This was carried out according to the manufacturer's recommended protocol: end repairing, adaptor ligation, size selection, amplification, purification and sequencing. As for computational analysis, we resorted to a variety of third party software. These include bowtie, tophat, cufflinks, and IGV; while the hardware requirements were met by an Amazon cloud-computing platform.

A total of five different samples have been completed thus far. A wealth of valuable information regarding gene structure, transcript prevalence and transcriptome dynamics is now available to assist in furthering our understanding of development. Previously, most gene models were based on in silicon prediction. This approach leaves much to be desired and has been found to be problematic. By using a data-driven approach, we have now circumvented most of the issues that arise from prediction algorithms. We have generated a highly precise definition of gene models from these RNA-seq results. Particularly interestingly, we found abundant cases revealing alternative promoters and multiple splice forms. This information is invaluable for accurate gene regulatory studies. Furthermore, comparison among technical replicates indicates that RNA-seq is capable of measuring transcript prevalence precisely. Utilizing a random simulation with regards to the amount of sequence reads, we established that 20 million reads is sufficient to measure transcripts as low as ~100 copies per embryo.

Currently, we have approximately 15 more samples being processed, and are improving the computational analysis pipeline.

19. Comparative study of *delta* gene *cis*-regulatory modules across 500 million years of evolution

Feng Gao

The sea star *Patiria mineata* and the sea urchin *Strongylocentrotus purpuratus* last shared a common ancestor about a half a billion years ago. Since *cis*-regulatory analysis can be done in both animals using comparable methods of gene transfer into eggs, the opportunity exists for decisive exploration of the evolutionary mechanism by which expression of given developmental genes has been redeployed. An example is the *delta* gene, encoding a Notch ligand. Previous work showed that there are two sharp differences in the way this gene is expressed: in sea urchins *delta* is transcribed in the skeletogenic micromere lineage while in sea stars there is no such lineage. In consequence, in sea urchins the result of this *delta* expression is an essential activation of mesoderm genes, and there is no Notch signal input into the endoderm, while in sea stars, delta expression in mesoderm is instead essential for endoderm specification. Other loci of *delta* expression, in what are possibly neuronal cells embedded in the ectoderm, and in non-skeletogenic mesoderm, are shared. The *cis*-regulatory system controlling skeletogenic and non-skeletogenic mesodermal expressions in sea urchins was previously described. Using BAC *in vitro* recombineering, and a high throughput *cis*-regulatory analysis system, we have isolated and studied the organization of the *Patiria delta cis*-regulatory system. A *cis*-regulatory module was isolated that recapitulates *delta* expression in the *Patiria* ectoderm, and within this region Hesc and Runx sites were functionally verified by site mutation. But paradoxically, these are inputs that control *delta* expression in the non-skeletogenic mesoderm of the sea urchin. Thus, when introduced into sea urchin eggs, this construct expressed GFP was expressed in mesoderm and ectoderm. In this case, therefore, it is the *cis*-regulatory apparatus which is conserved, probably having a common origin in the common ancestor of both species, but the gene is expressed differently because in the lineage leading to sea urchins, the *trans* regulatory landscape this module responds to has been co-opted to the mesoderm. We are now trying to determine whether the *Patiria* mesodermal module is expressed in the sea urchin skeletogenic lineage, or anywhere, so as to approach the problem of the mechanism by which derived the sea urchin skeletogenic expression system evolved.

20. Specification of the skeletogenic cell lineage in the sea urchin *Eucidaris tribuloides*

Eric Erkenbrack

The primary mesenchyme cell lineage of the purple sea urchin *Strongylocentrotus purpuratus* is as an important model for understanding cell lineage specification, cell signaling and, more broadly, general principles of developmental gene regulatory networks (GRNs). Primary mesenchyme cells (PMCs) are autonomously specified, precociously ingress into the blastocoel before vegetal plate invagination and eventually give rise to the larval skeleton. PMC specification has been shown to be under the control of the *hesC-pmar1* double-negative gate. Pmar1, a repressor that is activated in response to nuclearized TCF-β-catenin and Otx, prevents the ubiquitous repressor HesC from being expressed in the micromeres. Genes important to skeletogenic specification, including *alx1*, *ets1/2*, and *t-brain*, are then activated in the large micromeres in the absence of HesC repression.

S. purpuratus belongs to the lineage of echinoderms known as the euechinoids, most of which produce a larval skeleton by way of a precociously ingressing cell lineage. Approximately 250 million years removed from the euechinoids are the cidaroid urchins, many of which produce a larval skeleton by wholly different means. In the cidaroid sea urchin *Eucidaris tribuloides*, the larval skeleton arises from cells that delaminate during gastrulation from the tip of the archenteron. *E. tribuloides* lacks a precociously ingressing cell lineage and, given evidence from the fossil record, most likely represents the ancestral state of larval skeletogenic specification in the class Echinoidea. Interestingly, preliminary data suggest that *pmar1* is absent from the transcriptome of *E. tribuloides*. Furthermore, *pmar1* has, to date, not been found in the transcriptomes of two distantly-related clades of echinoderms—asteroids and ophiuroids—which suggests that it may be a euechinoid novelty.

To more fully understand the evolution of GRNs in development and the evolution of embryonic morphologies in general, this project aims to elucidate the molecular mechanisms underlying the specification of the skeletogenic cell lineage in *E. tribuloides.* Disentangling these GRNs will afford us unique and substantive insight into the origin and evolution of the euechinoid skeletogenic cell lineage.

21. The sea urchin genome database SpBase

R. Andrew Cameron, Qiu Autumn Yuan, Dong He, Ung-Jin Kim, Manoj Samanta, David Felt

The sea urchin genome database system organizes, classifies and publishes various kinds of information derived from the genome sequence and gene models of the purple sea urchin *Strongylocentrotus purpuratus*, as well as allied species from which useful genome sequence comparisons can be made. The sequences have been generated by various individual sequencing projects and the major genome sequencing efforts of the Sea Urchin Genome Sequencing Project as managed by the Baylor College of Medicine, Human Genome Sequencing Center (BCM-HGSC). The efforts at annotation at SpBase take advantage of well developed Best Practices in biological curation and bring the special knowledge of the sea urchin research community to bear on this information. The advent of next-generation short read sequencing of transcriptomes promises to provide a new and vast source of expressed sequence data to include in our analyses and postings. As the permanent home for manual annotations and other meta-information that can be associated with the genome sequence (including expression data and array analyses), the incorporation of these new datasets require continual adjustment in the varied sequence collections we add.

SpBase consists of several PostgreSQL databases and the web page code needed to access them. Text information of various kinds in support of the distribution of Sea Urchin Genome Resource materials is mounted as well. The databases are: 1) a sequence coordinate database using the Chado schema from the GMOD project. This dynamically drives the Gbrowse sequence assembly viewer. 2) An annotation database that organizes gene annotation data linking expression in formation and gene structure to various compiled searches of protein motifs, etc. About 21,000 of the gene models in the Official Gene Set (OGS) have some amount of information posted. We have begun to construct another sequence database that will house the expressed sequence tags and next-generation short read sequencing of transcriptomes. Because the genome sequence is a mosaic of two haplotypes and may differ from individual expressed sequences this data structure is needed for primer design and other molecular biology procedures.

The large data sets focused on gene expression that are detailed above represent a very real problem in organization, presentation and display that are quite separate from the annotation work that must be done. Indeed, there is a significant backlog of expression data in the literature and on public web sites already. For example, datasets from the NanoString nCounter utilize a codeset of 172 regulatory genes from the sea urchin embryo and thus, produce 172 transcript numbers in each run. Together, these datasets constitute an inventory on which to start working. The questions are obvious: Can temporal expression values from different measurement methods be productively compared in a non-confusing display? How can 3D spatial information be efficiently rendered so an observer can use it? Is there a way to automate these renderings for 20,000 genes? All of these questions are being addressed through the construction of several new experimental data pipelines. Since excellent software is already available for the presentation of this information, the iterative process of design, test implementation and redesign is expected to be rapid. Early versions of the code are now in working order and they will be mounted on a development version of the website and reviewed by a volunteer group of

experimentalists. We will refine these pipelines and incorporate them into the public version.

It should be noted that the accumulation of transcript sequence in turn serves as a basis for refining the predicted transcripts. We are actively incorporating these new data as they arrive. In the process, we continue to resolve the inconsistencies and gaps in the data structures resulting from the uneven practices of the manual annotators working on the original gene set.

22. Improving the *Strongylocentrotus purpuratus* genome sequence assembly

R. Andrew Cameron, Manoj Samanta, Dong He

In addition to the first 500 million Solid mate pair reads generated from the reference sea urchin genomic DNA last year, another similar amount has been generated this year. The combined reads were used to improve the version 2.1 genome sequence assembly by the BCM-HGSC. Using a specially modified version of the Atlas assembler program, a new assembly, version 2.5, was produced. The number of scaffolds was reduced from 114,222 in version 2.1 to 77,726 in 2.5. The smallest sized fragment included in the portion of large fragments that comprised 50% of the bases (N50) is 166,504 bp, a significant increase from 123,485 bp in version 2.5. A manual scan of the changes in the assembly showed that a large number of changes were small scaffolds that were fitted in to the previous gaps in scaffold assemblies. The SpBase staff has mapped all of the sequence features to the new assembly in preparation for the next build of the data base to be released in July 2010. In addition to an improved genome sequence assembly, the new build will remove many inconsistencies in data curation that remained in the previous version.

23. Further annotation of the sea urchin gene set

R. Andrew Cameron, Qiu Autumn Yuan, Ung-Jin Kim

The great majority of predicted gene models in *S. purpuratus* have been identified by protein sequence homologies to putative orthologs in various non-sea urchin species. Approximately 21,000 gene models have been annotated from the original collection of 28,944 GLEAN3 gene models. The least evidence for an annotation is appositive result in an electronic, automated computational process. Here, each of the predicted proteins is queried by BLAST against nonredundant protein set at the NCBI protein database, followed by visual analysis of the graphic output. Conserved protein domains and ortholog-like proteins are identified, and putative gene identity is assigned to the queried gene model after carefully inspecting the extent (span) of the matches and the degree of homologies between query and target sequences. Many genes have in addition support from expressed sequence tags or manual comparisons to experimental evidence of various sorts. More than 7,000 of the models show strong similarity to ESTs. Unfortunately the range of stages and tissues queried for ESTs is rather small. Thus, many real genes do not have support. Furthermore, the tiling array data and ESTs in combination map to regions of the genome not covered by predicted gene models suggesting the presence of undocumented genes. We expect that the range of next-generation short-read sequencing of transcriptomes will fill in the needed evidence for expressed genes. We are currently revising the evidence hierarchy for gene annotation and we will include such values in the annotation data we present.

One short coming of gene predictions is the rather poor capacity to discern un-translated regions. We have already derived 3' UTR sequences from tiling array data and mapped them onto the version 2.5 genome. These data are now included in our annotations and in the gene tracks for viewing in Gbrowse. These 3' UTR sequences are now being corrected by comparison to EST data as well.

24. Network gene annotation project

Qiu Autumn Yuan, R. Andrew Cameron

One of the strengths of the sea urchin embryo research model is the ease with which one can describe gene regulatory networks. As this experimental approach matures it is becoming clear that a gene annotation knowledge base is indispensible for designing experiments. Gene sequences, expression patterns and responses to perturbation are the central classes of information to be used here. Our previously built and currently maintained database of sequence and expression data for sea urchin regulatory genes fills that role. 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. Both the new ectoderm regulatory network study and a more precise spatial expression analysis in the endomesoderm from the Davidson laboratory have added a unique chunks of data to this effort. These unpublished data are now publically available through a Caltech web site.

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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 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, as in our recent studies of the genetic competence system of *Bacillus subtilis* (Süel *et al*., 2006; Süel *et al*., 2007).

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), as well as our studies of frequency modulated nuclear localization bursts in yeast (Cai *et al*., 2008). Most recently, we have analyzed signaling through the Notch pathway in and between individual mammalian cells. This work showed that same-cell (cis) interactions between Notch and Delta lead to a situation where individual cells can 'send' or 'receive' signals, but cannot do both at the same time.

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. Recently, we have examined the role that noise plays in enabling an alternative mode of evolution through partially penetrant intermediate genotypes (Eldar *et al*., 2009). We have also studied the way in which dynamic correlations of fluctuations in gene network dynamics can help identify active regulatory interactions (Dunlop *et al*., 2008).

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.

25. Investigating stem cell-specific regulatory interactions in single cells

Julia Tischler

Embryonic stem cells have the capacity to both self-renew and to differentiate into cellular derivatives of all three germ layers (pluripotency). To date, numerous studies have identified specific genes and regulatory interactions that play a central role in maintaining pluripotency and controlling differentiation into specific cell fates. In addition, recent research has shown that stem cell populations are inherently heterogeneous, displaying substantial cell-to-cell variability in both the expression of specific regulatory genes and in the propensity to differentiate. These results raise two fundamental and inter-related questions: First, how does the underlying genetic regulatory circuitry generate and regulate heterogeneity in the ES cell state? Second, how does this heterogeneity functionally modulate the propensity of a cell to remain pluripotent or differentiate into specific fates? In order to address these questions, it is critical to

analyze the gene expression and dynamics of stem cell-specific regulators at the single-cell level. We are combining various single-cell and time-lapse techniques to address these questions. Results will provide a dynamic picture of gene expression variability in the cell, and how that variability directly impacts cell states and fate decisions. Understanding stem cell-specific regulatory dynamics will be critical for applications in regenerative medicine and for developmental biology more generally.

26. Signal regulation of stem cells

Frederick E. Tan

Although developmental decisions are enacted at the single cell level, it remains unclear how cell state and cell-extrinsic signals are integrated to determine cell fate. Mouse embryonic stem (mES) cells rely on intercellular signaling to maintain pluripotency. Even when known signaling ligands have been removed from media, chemical inhibitors of the corresponding pathways still impact cell fate decisions, highlighting an intricate relationship between differentiation, pluripotency and signaling activity. To begin to explore how intercellular signals influence developmental potential, we probe the relationship between signaling and pluripotnecy. We are employing different techniques including mass spectrometry, two-dimensional gel electrophoresis, microarray analyses and time-lapse movies for this purpose. These studies will permit a better understanding of how developmental potential is maintained at the single cell level.

27. Global regulatory dynamics of *B. subtilis* stress response

James Locke, Jon Young, Michael B. Elowitz

Even genetically identical cells grown in the same environment are frequently found to exhibit heterogeneous 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. A variety of single-cell studies are now revealing a rich and varied structure to the transcriptional state space of *B. subtilis*. We are developing mathematical models to explain and characterize this variability from a network perspective. In particular we are using the general stress response sigma factor sigB as a model system to understand how heterogeneous states can be generated.

28. Analysis of Notch-dependent pattern formation processes

Amit Lakhanpal, David Sprinzak, Lauren LeBon, Jordi Garcia-Ojalvo

Developmental patterning requires juxtacrine signaling in order to tightly coordinate the fates of neighboring cells. Recent work has shown that Notch and Delta, the canonical metazoan juxtacrine signaling receptor and ligand, in the same cell (in *cis*) mutually inactivate each other. This interaction generates mutually exclusive sending and receiving states in an individual cells. It generally remains unclear, however, how this mutual inactivation interaction and the resulting switching behavior impact developmental patterning circuits. Here we address this question using mathematical modeling of two canonical pattern formation processes: boundary formation and lateral inhibition. We find that mutual inactivation can form sharp boundaries along the *Drosophila* wing vein with robustness to a specific profile of noise, and forms boundaries under biologically-relevant conditions that cannot be accommodated by models lacking mutual inactivation. Mutual inactivation speeds up lateral inhibition patterning dynamics, removes the need for cooperative regulatory interactions, and increases the robustness (range of parameter values) that permit pattern formation, compared to canonical models. Furthermore, mutual inactivation enables a simple lateral inhibition circuit architecture that requires only a single downstream regulatory step. Together, these results suggest that mutual inactivation in general simplifies the architecture of patterning circuits by encoding a rapid difference-promoting logic in the signaling system itself, reducing the number of regulatory levels required for their operation. It thereby enables robust patterning processes that would be difficult or impossible to implement in its absence. These results provide a framework for analysis of more complex Notch-dependent developmental systems.

29. Sporulation initiation dynamics in *B. subtilis*

Joseph H. Levine, Shaunak Sen, Michael B. Elowitz

We are investigating the initiation of sporulation in the genetic model bacterium *Bacillus subtilis*. In response to various stress conditions, *B. subtilis* initiates expression of sporulation initiation genes in a highly noisy and heterogeneous manner. This gene expression continues for multiple cell cycles until the cell eventually begins morphological transformation into a resistant spore. Gene expression is regulated by the master regulator of sporulation, Spo0A, a two-component response regulator. Spo0A is embedded in an elaborate signal transduction cascade, and also feeds back to both positively and negatively regulate its own expression and phosphorylation state. We are working to ascertain the genetic mechanisms behind these heterogeneous gene expression dynamics using quantitative time-lapse microscopy and specific rewiring and genetic perturbations to the phosphorelay circuit.

30. Functional interrogation of the transcriptional network underlying pluripotency

Zakary Singer

Many of the key regulatory components (transcription factors and signaling pathways) and regulatory interactions responsible for maintaining pluripotency and controlling differentiation of ES cells have now been identified [Chen, *Cell*, 2008; Macarthur, *Nat. Rev. Mol. Cell Biol.*, 2009]. However, it remains generally unclear how this network functions at the level of individual cells. What is needed is a way to determine the quantitative relationship between levels of specific regulators and expression of their targets. To do this, we use quantitative time-lapse microscopy techniques [Sprinzak, *Nature*, 2010] that allow direct analysis of regulatory interactions in individual cells. By placing transcription factors under the control of an inducible promoter, we are able achieve a wide range of exogenous production levels. At the same time we can keep this transgene shut off and then induce it suddenly during a movie, allowing precise control of its expression. When coupled with GFP reporters of endogenous transcriptional activity of individual genes, one can observe the resulting temporal dynamics of targets of this perturbation, allowing for a functional understanding of these transcriptional networks.

31. Partial penetrance facilitates developmental evolution in bacteria

Avigdor Eldar, Vasant Chary, Panagiotis Xenopoulos, Michelle E. Fontes, Oliver C. Loson, Jonathan Dworkin, Patrick Piggot, Michael B. Elowitz

Development normally occurs similarly in all individuals within an isogenic population, but mutations often affect the fate of individual organisms differently. This phenomenon, known as partial penetrance, has been observed in diverse developmental systems. However, it remains unclear how the underlying genetic network specifies the set of possible alternative fates and how the relative frequencies of these fates evolve. We identified a stochastic cell fate determination process that operates in *Bacillus subtilis* sporulation mutants and showed how it enables the gradual evolution of a novel developmental fate through states of intermediate penetrance. Mutations that attenuate cell-cell signaling produced a set of discrete alternative fates not observed in wild-type cells, including the relatively infrequent formation of two viable "twin" spores, rather than one, within a single cell. We systematically tuned the penetrance of each mutant fate by genetically perturbing cellular processes such as chromosome replication, septation, and signaling. In particular, we obtained strains that formed twin spores at high penetrance. These strains exhibited similar features to natural twin sporulation in the anaerobic spore-former *Clostridium oceanicum*, suggesting that they could represent evolutionary intermediates. A mathematical model showed how twin sporulation can evolve through gradual changes in fate penetrance, and how specific experimentally observed features of the system facilitate this transition. Together these results show how noise can facilitate developmental evolution by enabling the initial expression of discrete morphological traits at low penetrance, and allowing their stabilization by gradual adjustment of genetic parameters.

In a follow-up to this work, we are studying the natural development and evolution of twin sporulation, which may have evolved through genetic assimilation on frequency. Using RNA FISH we will follow gene expression pattern of the naturally twin-forming species, *C. oceanicum*. We have sequenced the bacteria and are currently characterizing genes that may explain the formation of twins under some conditions and the formation of mono-spores under other conditions. We hope that this research into the environmental plasticity of twin frequency will shed more light on its evolution and the relation between partial penetrance, environmental plasticity and evolution.

32. *Cis* interactions between notch and delta generate mutually exclusive signaling states

David Sprinzak, Lauren LeBon, Amit Lakhanpal, Michael B. Elowitz

The Notch-Delta signaling pathway is the canonical pathway for communication between neighboring cells during development. It plays a critical role in the formation of "fine-grained" patterns, generating distinct cell fates among groups of initially equivalent neighboring cells, and in sharply delineating neighboring regions in developing tissues. The Delta ligand has been shown to have two activities: it trans-activates Notch in neighboring cells, and cis-inhibits Notch in its own cell. However, it remains unclear how Notch integrates these two activities and how the resulting system facilitates pattern formation. To address these questions, we developed a quantitative time-lapse microscopy platform for analyzing Notch-Delta signaling dynamics in individual mammalian cells. By controlling both cis- and trans-Delta levels, and monitoring the dynamics of a Notch reporter, we measured the combined cis-trans input-output relationship for the Notch-Delta system. The data revealed a striking difference between the response of Notch to trans- and cis-Delta: While the response to trans-Delta is graded, the response to cis-Delta exhibits a sharp, switch-like response at a fixed threshold, independent of trans-Delta. We developed a simple mathematical model that shows how these behaviors emerge from the mutual inactivation of Notch and Delta proteins. This interaction generates an ultrasensitive switch between sending (high Delta / low Notch) and receiving (high Notch / low Delta) signaling states. Critically, the two states are mutually exclusive: cells predominantly send or receive, but cannot do both simultaneously. At the multicellular level, this switch can amplify small differences between neighboring cells even without transcription-mediated feedback. This Notch-Delta signaling switch facilitates the formation of sharp boundaries and lateral inhibition patterns in models

of development, and provides insight into previously unexplained mutant behaviors.

33. Nuclear localization bursts and signal encoding in yeast

Chiraj Dalal, Long Cai, Kasra Rahbar, Michael Elowitz

In yeast, the transcription factor Crz1 is dephosphorylated and translocates into the nucleus in response to extracellular calcium. Using time-lapse microscopy, we found that Crz1 exhibited short bursts of nuclear localization (~2 minutes) that occurred stochastically in individual cells and propagated to the expression of downstream genes. Strikingly, calcium concentration controlled the frequency, but not duration, of localization bursts. Using an analytic model, we found that this frequency modulation (FM) of bursts ensures proportional expression of multiple target genes across a wide dynamic range of expression levels, independent of promoter characteristics. We experimentally confirmed this theory with natural and synthetic Crz1 target promoters. Another stress response transcription factor, Msn2, exhibits similar, but largely uncorrelated, localization bursts under calcium stress. These results suggest that FM regulation of localization bursts may be a general control strategy utilized by the cell to coordinate multi-gene responses to external signals.

In this work, we observed that the yeast transcription factor Crz1 exhibits frequency-modulated bursts of nuclear localization in response to its stimulus, calcium. This behavior enables proportional regulation of Crz1 target genes in response to calcium, revealing a novel regulatory strategy cells may use to optimally tune expression levels of groups of promoters (Cai *et al*., 2008). These observations provoke the question of what other related regulatory strategies might be employed by the cell. Taking advantage of the yeast GFP library, we are using high-throughput time-lapse microscopy to identify other localization dynamic regulatory systems.

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Research & Laboratory Staff: Natasha Bouey, Sonia Collazo, Jeffrey Fingler, Leigh Ann Fletcher, Mary Flowers, Kristy Hilands, David Huss, Aura Keeter, Edriss Merchant, Dan Pacheco, Ilana Solomon*, Jayne Sutton, Sarah Sweeney
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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. Our approach has been to develop imaging tools that are sufficiently robust so that the movements, lineages and gene expression in living cells can be analyzed as an adjunct to the techniques more typically employed by systems biology. Systems biology provides a means to organize the explosion of data from molecular approaches and the dramatic progress from *in vitro* culture assays, but it 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 imaging fast events in the developing embryo, ranging from the movements of cilia in the developing ear and left-right organizer and the flows these cilia generate, to the workings of the beating heart as it takes shape. This has required the development of new image acquisition and processing tools that offer quantitative analyses of the key events. These tools are proving to be generalizable to other systems such as the *Drosophila* embryo. As a result, we have great hope for exploring events that have previously been too difficult to address.

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 and collecting 4D light microscopic 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.

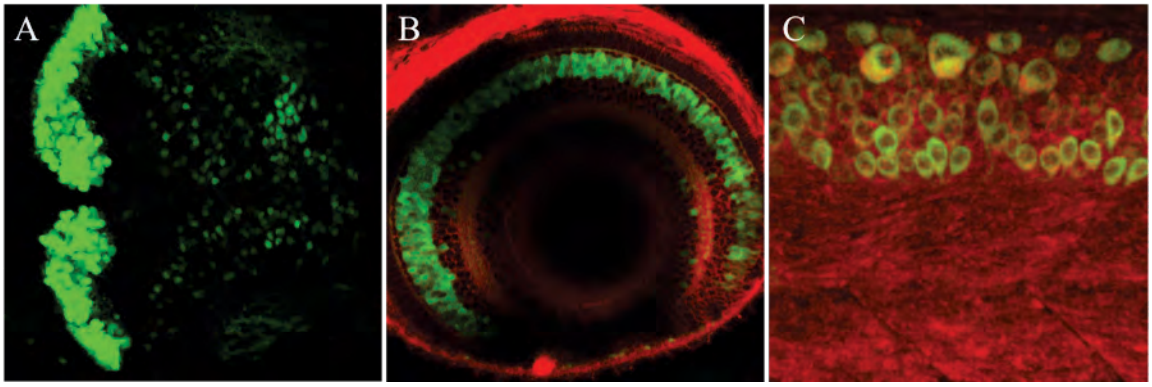
34. Screening for developmental expressed genes by FlipTrap

Le A. Trinh, Tatiana Hochgreb, Chathurani Jayasena, Frederique Ruf, Ankur Saxena, Rasheeda Hawk, Aidyl Gonzalez-Serricchio, Alana Dixson, Ho-Yin Leung, Ilana Solomon, Sean G. Megason, Marianne Bronner, Scott E. Fraser

A fundamental goal in biology is to identify genes involved in biological processes and study their protein function. To this end, we have developed a gene trapping approach to generate endogenously expressed fluorescent fusion proteins and create Cre-mediated conditional alleles at the same loci. The gene trapping vector, termed FlipTrap, consists of an artificial exon encoding the yellow fluorescent protein, citrine, flanked by a splice acceptor and donor in the forward orientation, the red fluorescent protein, mCherry and a polyadenylation signal in the reverse orientation, and two pairs of heterotypic *lox* sites. Insertion of the citrine exon into the intron of an actively expressed gene by Tol2 transposition leads to the splicing of the citrine sequence into frame with the endogenous mRNA and hence, the formation of a full-length functional fluorescent fusion protein. We have used the FlipTrap vector to screen for developmentally expressed genes in zebrafish by integrating the FlipTrap vector throughout the genome of zebrafish. To date, we have generated a total of 241 lines through screening the progeny of F0 fish that have been injected with the FlipTrap construct. Of these, 79 lines (32%) exhibit tissue specific expression. We have cloned 156 of the trap lines by 3'RACE analysis. By BLAST, 21% (n=33) of the 3' RACE clones sequences match genes of unknown function. These are genes that have been previously identified by EST screens but their functions have not been assessed. Our ability to create mutant alleles by Cre-lox recombination will allow us to study their function. In addition, 10% (n=16) of the 3'RACE clones show BLAST matches to unannotated genomic sequence and no known cDNA or ESTs indicating that we have trapped novel genes. Our ability to clone these sequences by 3'RACE indicates that these sequences correspond to genes that are actively transcribed and suggest that the FlipTrap is a viable alternative experimental approach for the identification of novel genes and can complement existing genomic efforts in the zebrafish research community.

In addition to fluorescent tagging, the FlipTrap construct has the ability to create conditional mutant alleles by Cre-lox recombination. In the FlipTrap, Cre-mediated recombination of heterotypic *lox* sites results in the excision of the citrine and splice donor sequence and "flipping" of the mCherry sequence into the forward orientation. This "flipped" allele leads to the expression of a truncated protein of the trap gene. We have two methods for introducing Cre recombinase into the FlipTrap lines; either by injection of *cre* mRNA into the single embryo or the use of transgenic lines that stably express Cre recombinase. We have developed a Cerulean-Cre fusion protein that allows us to track the expression of Cre recombinase in the embryo. Using the Cerulean-Cre fusion protein, we have generated transgenic lines that express Cerulean-Cre in specific tissues. These lines will allow us to perform tissue specific Cre-recombination of the FlipTrap lines.

We have performed Cre mutagenesis on a select number of trap genes that are ubiquitously expressed with unknown function using the tissue specific Cerulean-Cre transgenes. Of these, we have found that a FlipTrap of a coiled-coil domain containing a gene that has no known function, ct2a, exhibits defects in cardiac looping when mutated specifically, in the myocardium.



Confocal images of fluorescent protein expression (green) in FlipTraps in the cerebellum. (A), retina (B), and spinal neurons (C). Embryos B & C were counterstained with bodipyTR methyl ester (red).

35. Dystrophin mRNA transcripts localization using HCR in fliptrap zebrafish embryos

Frederique Ruf-Zamojski, Harry M. Choi, Scott E. Fraser*

Muscular dystrophies are a complex family of diseases leading to muscle degeneration. Duchenne muscular dystrophy is the most severe case, in which mutations in the dystrophin protein prevent links between the actin filaments and the extracellular matrix in muscle fibers. Zebrafish embryos have been shown as a potential model organism in which to study muscular dystrophies. One major goal would be to detect any problems in dystrophin as early as possible during development, possibly even at the RNA level once they start being expressed.

Although regular chromogenic *in situ* hybridization remains the gold standard for studying RNA expression in whole embryos (Fig. A), HCR enables us to achieve cellular resolution and look precisely at the localization of dystrophin mRNAs (Fig. B).

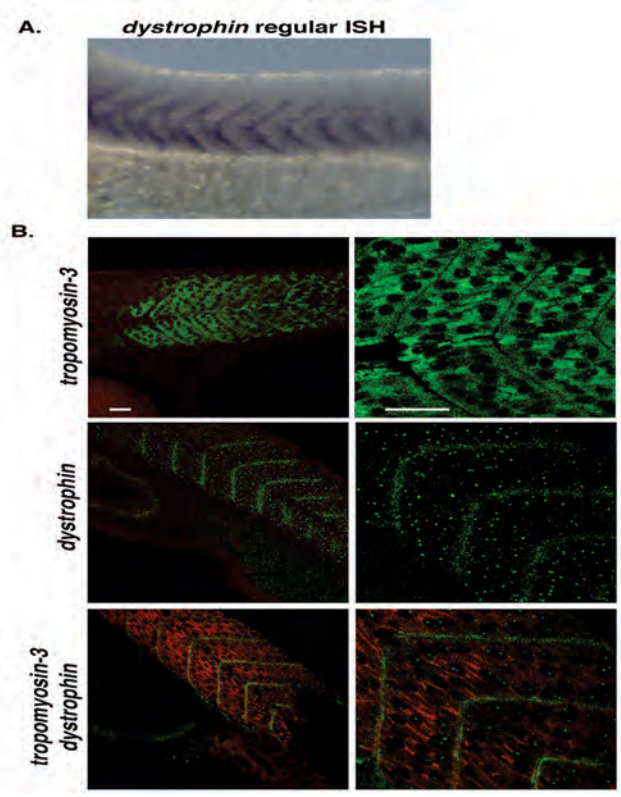


Figure: Localization of dystrophin mRNA in whole-mount wild-type zebrafish embryos using regular chromogenic *in situ* hybridization (A) and HCR (B). In B. zebrafish were labeled for dystrophin (green) and tropomyosin-3 (red) mRNAs.

Interestingly in the case of dystrophin mRNAs, HCR allowed us to detect nascent RNA transcript accumulation in the muscle cell nuclei. We studied how these transcription sites and overall dystrophin mRNA build-up are affected during development in wild-type versus muscular dystrophic morphants and mutants. These studies are still ongoing, but should bring interesting information on any possible variations between the different types of embryos.

**Niles A. Pierce's laboratory, Caltech*

36. Imaging somitogenesis and myogenesis in FlipTrap and muscular dystrophic zebrafish

Frederique Ruf-Zamojski, Sean Megason, Le Trinh, Scott E. Fraser

Somitogenesis and myogenesis are highly conserved mechanisms in vertebrate species that give rise to skeletal formations. These processes have not been investigated with single-cell resolution during the development of living embryos. It is important to observe these mechanisms with high resolution to understand how muscles form, are maintained, or degenerate, and to measure if there are any variations in single-cell behaviors when comparing wild-type and muscle-affected mutant development.

We are using a zebrafish transgenic line identified during a screen performed in our laboratory that has a trap in the dystrophin gene (Figure). Time-lapse microscopy imaging embryos using that zebrafish line enables us to look at cell movement during the somitogenesis process, as well as the expression of dystrophin in living and developing embryos. Using morpholino, we can knock-down dystrophin expression and see how the somitogenesis process is altered.

Individual cells in time-lapse movies are being tracked to see how knocking-down dystrophin early in development affects cell movements and somite formation. Early damages and variations might bring interesting information in cases of muscular dystrophies. Another part of the project will involve looking at the dynamics of other proteins as the muscles are forming and degenerating. This will be done using the different FlipTrap lines identified with protein expression in skeletal muscles.

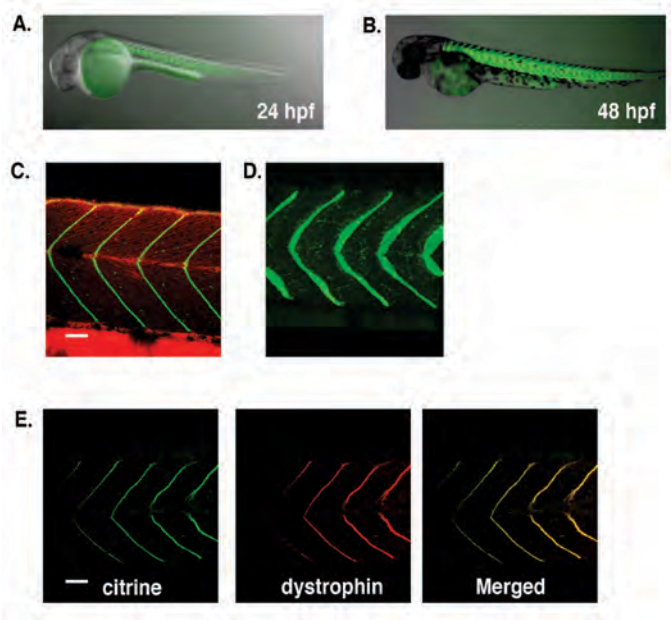


Figure: Dystrophin protein expression in dystrophin FlipTrap embryos. Expression of the dystrophin trap at 24 hpf (**A**) and 48 hpf (**B**) taken under a regular fluorescent scope. Green: citrine expression of the trap. The fluorescent image is overlaid on the brightfield image. (**C**) Confocal image of dystrophin expression in a single z-section through the myotome. Dystrophin (citrine, green), counterstain (Bodipy TR, red). (**D**) 3D projection of a 100 μm stack showing dystrophin (green) in the FlipTrap line at 48 hpf. (**E**) Comparison of dystrophin expression in the FlipTrap (left, citrine, green) versus antibody detection (middle, red). The merged image (right) shows perfect colocalization indicating that the trap is indeed showing endogeneous dystrophin protein localization.

37. It's alive! The persistent Rohon-Beard neuron in embryonic zebrafish

Alana Dixson, Deborah Marshall, Scott E. Fraser

Here we show that a subset of Rohon-Beard neurons (RB) are long-lived using the FlipTrap (FT) vector (Fig. 1) designed as part of the Center of Excellence in Genomic Science (CEGS) subgroup. RBs are large mechanosensory cells in the dorsal embryonic spinal cord of most vertebrates, including zebrafish (Fig. 2). Beginning at 24 hours post-fertilization (hpf) RBs reportedly undergo early apoptosis, and by 7 days post-fertilization (dpf) disappear entirely (Cole, 2001). As primary neurons, which are born first and establish their function during early embryogenesis (Kimmel, 1990), RBs enable sensation throughout the embryonic head and trunk (Sipple 1998). Until the mature Dorsal Root Ganglion (DRG) sensory system is established (Sipple, 1998) they provide an escape reflex and detect noxious stimuli by 20 hpf. Past researchers have shown that formation and maturation of the DRG is concomitant with RB elimination via apoptosis (Cole, 2001; Sipple, 1998; Williams, 00).

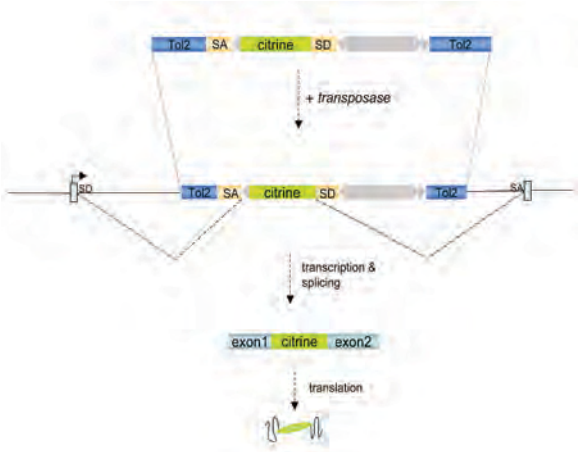
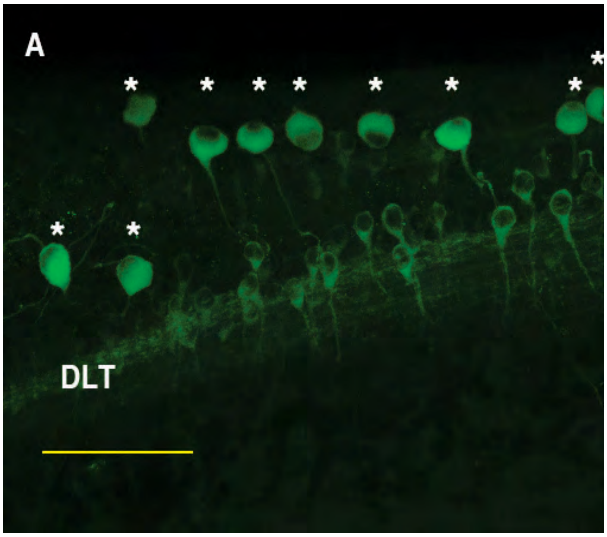


Fig. 1 Schematic of the *Tol2*-mediated protein-trapping strategy, courtesy of Le Trinh.



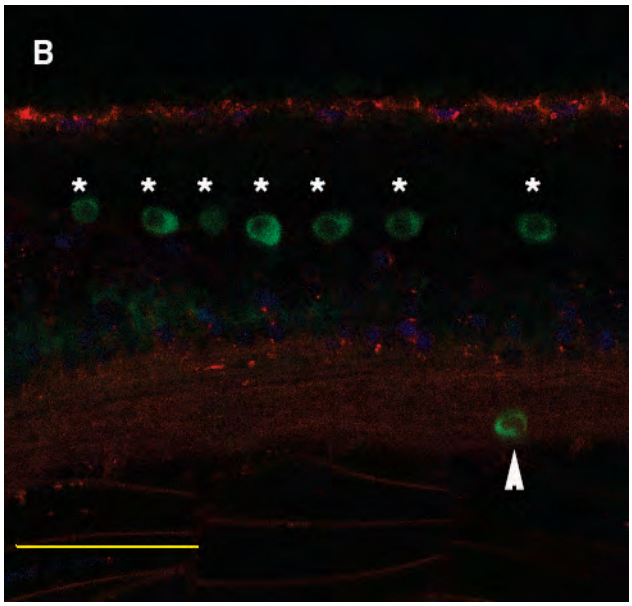


Fig. 2 Zebrafish Rohon-Beard neurons (RB) *in vivo* at A) 62 hpf, 3D projection, and B) 7 dpf. red = mRNA injection of m-cherry fluorescent protein localized to the membrane, blue = cerulean fluorescent protein localized to the H2B protein in the nucleus for automated segmentation of cells. In all images green = fusion protein, dorsal is up, caudal is to the right. Scale bar = 50 μm, * indicates RB neurons, arrowhead is DRG neuron, DLT = Dorsolateral tract. All images taken at 40X using the C-Apochromat 1.2 NA water immersion objective on the Zeiss Pascal or Exciter microscope.

The FT vector is a transposon-based gene trap that creates a fusion protein with citrine (YFP) to allow tracking of the endogenous temporal and spatial expression of developmentally important genes. In two FT lines, we used the fusion protein as a fluorescent marker of RBs. Through low-magnification, fluorescence microscopy from ~20 – 50 hpf, we found fusion protein expression spread from the Trigeminal Ganglion (TG) and several brain

regions to dorsal spinal cord neurons, including some RBs, and the lens. By 3-4 dpf a small number of DRG cells also expressed YFP. These fluorescent patterns persisted up to 30 dpf, beyond which we have no additional data (Fig. 3).

We performed immunohistochemistry to assess the molecular identity of the dorsal spinal cord cells in both FT lines (N = 200) in fixed animals. In parallel, we followed YFP-positive RBs for 30 dpf in whole animals *in vivo* via confocal laser scanning microscopy (CLSM). We confirmed that the large dorsal spinal cord cells are RBs and further characterized their location, morphology, and longevity beyond 7 dpf.

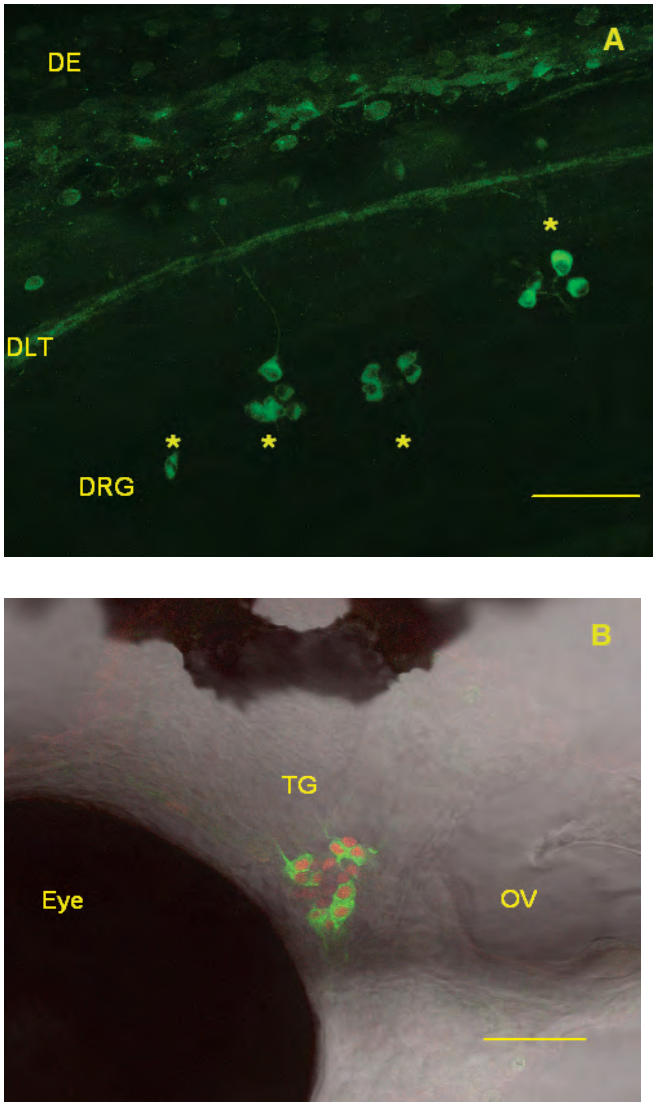


Fig. 3 A) DRG *in vivo* at 25 dpf, and B) Trigeminal ganglion (TG) at 3 dpf, red = Islet-1 staining to identify TG neurons. In all images green = fusion protein, dorsal is up, caudal is to the right. Scale bar = 50 μm,* indicates DRG, DLT = Dorsolateral tract, DE = dorsal ectoderm, OV = otic vesicle. All images taken at 40X using the C-Apochromat 1.2 NA water immersion objective on the Zeiss Pascal or Exciter microscope.

38. Origin and migration of Gabaergic cells that differentiate, proliferate, and migrate into craniofacial structures

Rasheeda Hawk, Eduardo Rosa-Molinar, Scott E. Fraser*

Neural crest cells found in the pharyngeal arches lead to the development of the bone and cartilage in the craniofacial regions (Yelick and Schilling, 2002). Current research suggests that GABA (A) expressing cells can regulate NCC proliferation through inhibitory events involving autocrine/paracrine signals. One of our FlipTrap transgenic fish lines, FT99b, begins demonstrating citrine protein expression in the olfactory bulb and telencephalon at 2 dpf. The gene was isolated by 3' race and identified as a GABA (A) receptor. Our goal is to investigate the involvement of GABA (A) cells on NCC proliferation by using bromo-deoxyuridine (BrdU) and 5-ethynyl-2' (EdU) cell proliferation assays. We will use BrdU or Edu to detect cells in S-phase in a pulse-chase approach. Proliferation usually occurs in ventricular areas in embryo and the pretectal areas of the zebrafish larvae. These are areas where we also find GABA (A)-citrine positive cells. We will also determine if the proliferating cells (mitotic cells) in the prectectal and preglomerular regions are also GABAergic cells by performing double labeling immunohistochemical staining to label both neural crest cells and GABA (A) cells.

Live imaging microscopy of embryos will be carried out using laser scanning confocal and two-photon microscopy (Megason and Fraser). A confocal microscope utilizes a point source to focus light down a cone and this allows the creation of several optical sections through a specimen. This information can then be converted into three-dimensional images of high resolution. Unlike confocal microscopy, two-photon microscopy uses infrared light and can thus, penetrate deeper within tissues. Because fluorescent excitation is mostly restricted to the focal point, this imaging modality causes very limited photo-damage and photobleaching to the specimen. Combining confocal and two-photon microscopy will thus, allow us to follow and fate map many components of neural crest cell migration and neurogenesis.

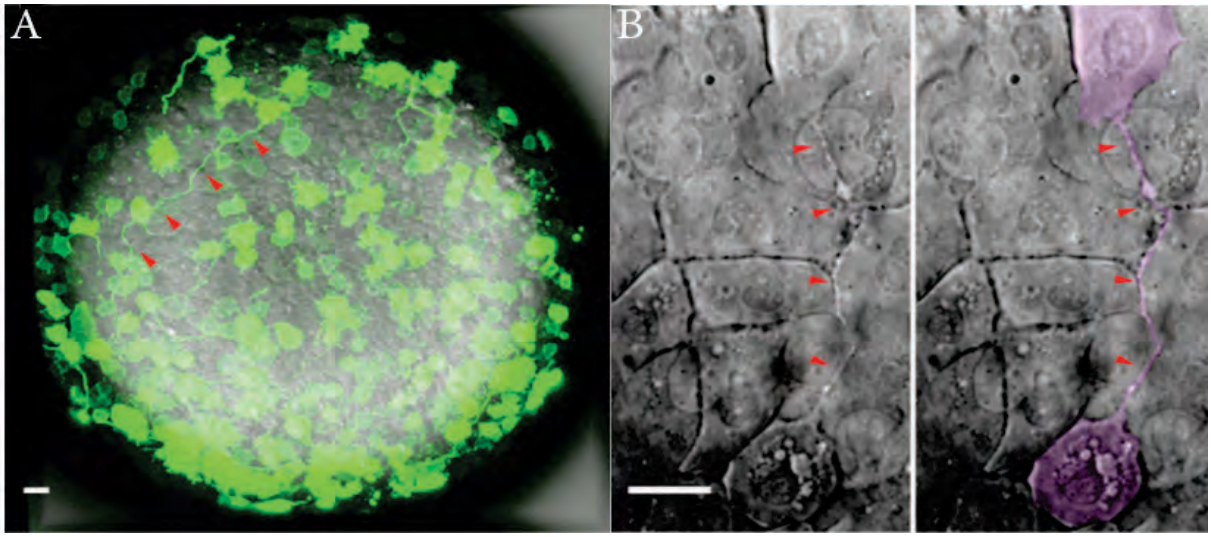
**University of Puerto Rico Rio Piedras, San Juan, PR*

39. Intercellular bridges In vertebrate gastrulation

Luca Caneparo, Periklis Pantazis, William Dempsey, Scott E. Fraser

The developing embryo is sculpted by progressive changes in cell shape, stereotypical cell movements and pattern events. A handful of *in vivo* studies have been focused on the cyto-architecture and the shape of cells in different germ layers during vertebrate gastrulation. To understand the full complexity of the germ layers and in particular of the epiblast, we highlight in a mosaic manner cells in the gastrula embryos using the photo-convertable protein Dendra2 with different membrane anchors. We show that a significant fraction of epiblast cells in the zebrafish embryo are endowed with intercellular bridges that reach several cell diameters in length and spread across different regions of the embryo throughout the gastrula stages.

The formation of these intercellular bridges occurs at pre-gastrula stages as a result of two dividing cells maintaining a membrane tether and moving further apart after mitosis is complete. Intercellular bridges connect via the plasma membrane distant pair of cells and exhibit a dynamic behavior along their plasma membrane. These findings refine the cellular landscape during gastrulation in the zebrafish embryo and need to be taken into account when cellular mechanics, cell-cell interactions or morphogenetic signaling is modeled and examined.



Cellular bridge visualization during gastrulation. (A) View of the entire animal pole of a mosaic zebrafish embryo expressing mDendra2 at the onset of gastrulation. (B) DIC image of an uninjected embryo showing two epiblast cells connected by an intercellular bridge (red arrow) and highlighted in the right part of the picture in magenta. Schematics are not to scale. Scale bar (A,B) 20 μm.

40. Two-photon light sheet microscopy

Thai Truong, Willy Supatto, David Koos, Scott E. Fraser

In recent years, biomedical research has benefited greatly from the emerging ability to acquire high-resolution, quantitative 4-dimensional (3 spatial dimension + time) image data of *in vivo* biological systems/processes. Standard, commercially-available, optical imaging technologies like point-scanning 1-photon confocal or 2-photon laser scanning microscopy (1p-LSM or 2p-LSM) allow collection of such data sets that cover volumetric samples with depth penetration of up to few hundreds of microns, at subcellular spatial resolution. These point-scanning techniques are inherently slow, however, since data is collected one point at a time. Also, the high numerical aperture focusing required for these techniques to give the subcellular resolution often create very high excitation light intensities, which produce photodamage effects in the live biological samples, limiting the range of applications for these techniques.

Light-sheet microscopy (LISM) is a century-old concept that has gained widespread recognition in recent years, due to its distinct advantages over standard LSM techniques in terms of imaging speed and photodamage. In LISM, a planar sheet of light is used to illuminate the sample, generating optical signal (such as fluorescence) over an optical section of the sample that is collected by a wide-field microscope camera oriented orthogonal to the light sheet (Figure 1). The orthogonal geometry between the illumination and detection pathways enables massive parallelization in both illumination and detection, therefore making LISM at least one order of magnitude faster than point-scanning LSM. Furthermore, the orthogonal geometry makes LISM much less photodamaging, since lower numerical aperture focusing is used (for the same image resolution as in standard LSM) and the sample is illuminated only one plane at a time (compared to the illumination passing through the entire sample in standard LSM). These features of higher speed and lower photodamage make LISM particularly suited for *in vivo*

imaging applications. One main limitation of standard LISM, however, is its limited depth penetration (how deep into a sample useful information can be collected), which is approximately the same as 1p-LSM, as in both cases the optically heterogeneous sample degrades the diffraction-limited spatial extent of the focused illumination light, reducing the resolution as one tries to image deep into the sample.

To improve the depth penetration of standard LISM, in the past year we have implemented LISM with 2-photon excitation, combining the benefits of LISM mentioned above with the high depth penetration of the 2-photon excitation process. We found that 2p-LISM achieves 2-fold improvement in depth penetration compared to standard LISM (Figure 2), rivaling that of 2p-LSM (which holds the record for the best depth penetration among all optical imaging techniques).

Also, we found that 2p-LISM is much less photodamaging than 2p-LSM, therefore allowing use of higher laser excitation power, yielding an order of magnitude improvement in imaging speed. These superior performance parameters of 2p-LISM have allowed us to image the entire embryonic development of the fruit fly *Drosophila*, at high enough spatial and temporal resolution to capture and distinguish a majority of all cells, without inducing any observable photodamage (Figure 3). Thus, 2p-LISM is a significant development for us towards the goal of in toto imaging of embryonic development, and we are in the process of applying this new imaging modality to various biological imaging applications.

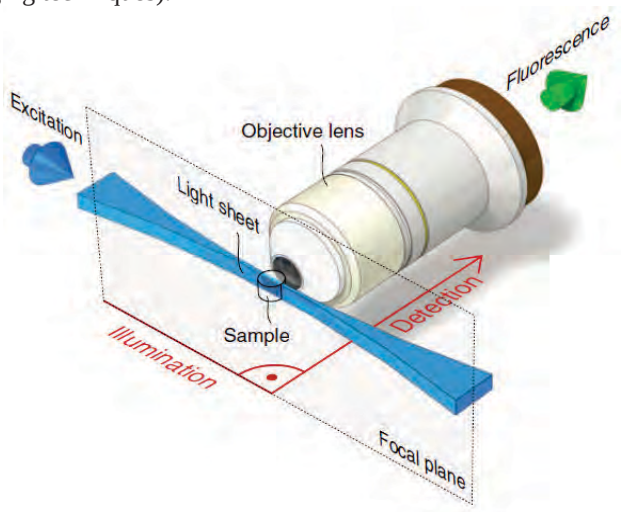


Fig 1. Schematic of light-sheet microscopy. The illumination and detection optical paths are orthogonal (unlike in standard microscopes, where they are collinear). Figure is from J. Huisken and D.Y.R. Stanier, *Development* (2009) **136**:1963-1975.

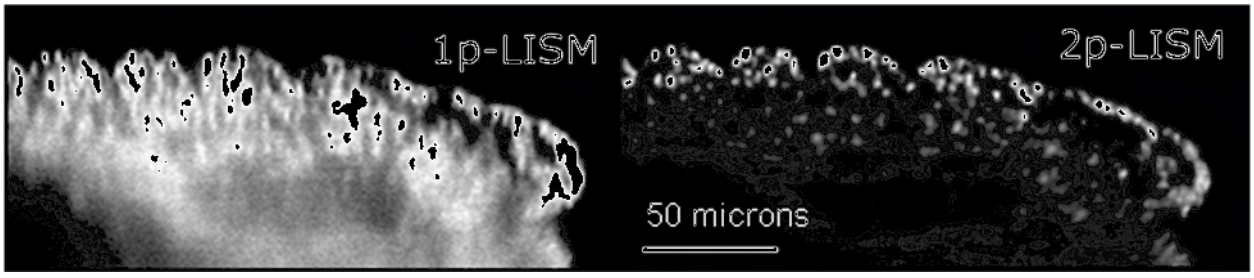


Fig 2. Superiority of 2-photon (right) over 1-photon (left) excitation in imaging depth penetration of light sheet microscopy (LISM) for 3D samples. Shown are fluorescence images of the same meridional plane inside a live H2A-GFP (nuclear-labeled) *Drosophila* embryo. Individual cell nuclei, denoted by bright dots, are resolved deep inside the sample only with 2-photon excitation.

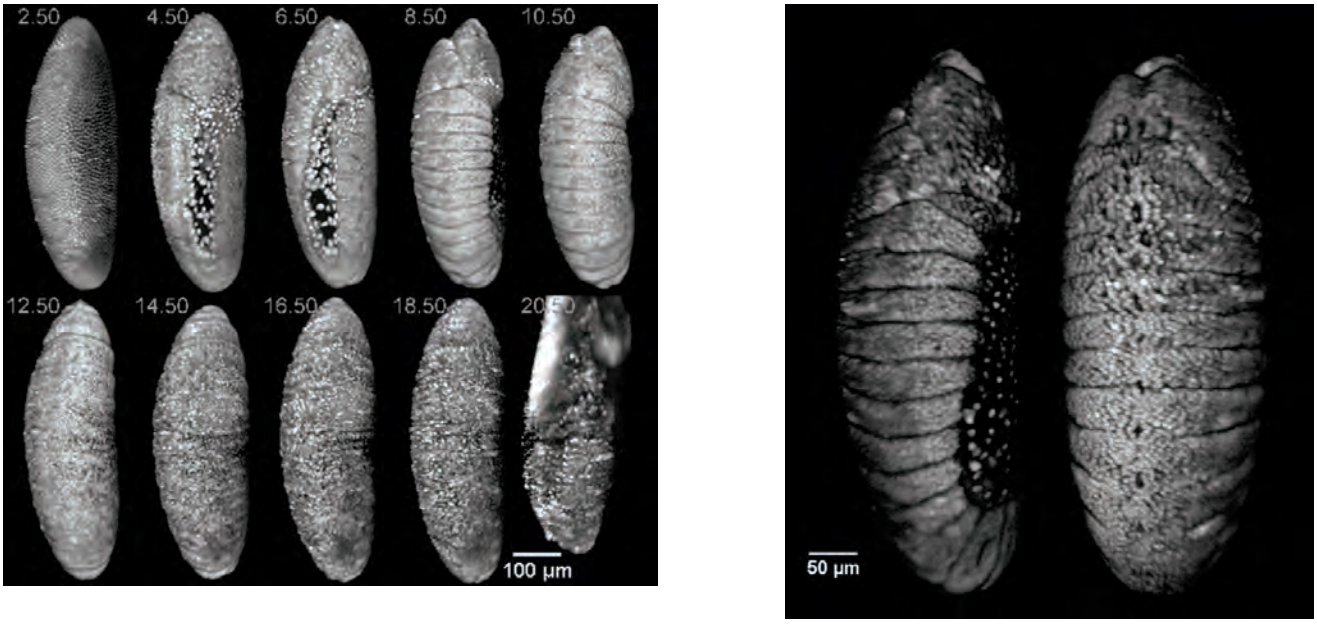


Figure 3: Entire *Drosophila* embryonic development imaged with 2-photon light sheet microscopy. The left figure shows a lateral-view 3D reconstruction of an H2A-GFP (nuclear-labeled) embryo imaged from about 2.5 hours post fertilization (hpf) to when it hatched and crawled away at about 20 hpf. The imaging was done with our home-built 2-photon light sheet microscope, where full volume scans with voxel size of approximately 1 micron^3 were taken at time resolution of 1 minute, yielding more than a thousand time points for the 17.5-hours time lapse. Only 10 representative time points are shown above in the left panel, with the number next to each image corresponding to the age of the embryo in hpf. The right panel shows the zoomed in view of the embryo at about 8 hpf, showing the lateral (left) and ventral (right) views. A majority of all cell nuclei are captured and clearly visualized. By observing the cellular morphogenetic movements and patterns, and by comparing with control embryos mounted in the same imaging chamber, we verified that all imaged embryos (n=5) developed normally.

41. Second harmonic generation (SHG) nanoprobes for *in vivo* imaging

Periklis Pantazis, James Maloney, David Wu, Scott E. Fraser

Fluorescence microscopy has profoundly changed how cell and molecular biology is studied in almost every aspect. However, the ultimate need of characterizing biological targets is largely unmet due to fundamental deficiencies associated with the use of fluorescent agents. Dye bleaching, dye signal saturation and tissue autofluorescence can severely limit the signal-to-noise ratio (SNR). Here, we demonstrate that second harmonic generating (SHG) nanoprobes are suitable for *in vivo* imaging and eliminate most of the inherent drawbacks encountered in classical fluorescence systems. These nonlinear nanocrystals of less than 100 nm are capable of generating second harmonic signals because they do not possess an inversion symmetry and can be detected by conventional two-photon microscopy. Unlike commonly used fluorescent probes, SHG nanoprobes neither bleach nor blink, and the signal does not saturate with increasing illumination intensity. SHG nanoprobes provide a superb SNR in live imaging of zebrafish embryos, and unlike endogenous second harmonic generation their signal can be readily detected both in trans- and in epi-direction**.**

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

Periklis Pantazis, Nathan Hodas, 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.

43. Endogenous second harmonic generation in developing zebrafish skeletal muscle

Periklis Pantazis, William Dempsey, Scott E. Fraser

Duchenne Muscular Dystrophy (DMD), which results from a deleterious mutation in the muscle structural protein dystrophin, affects 1 in 3000 male individuals. Current methods of diagnosis of neuromuscular disorders such as DMD are invasive and are only performed after a child shows signs of reduced muscle function, often after significant damage has already compromised much of the muscle tissue. In this study, we use second harmonic generation imaging (SHGi) to monitor the progression of early muscular development in live zebrafish, a vertebrate species with comparable muscle anatomy to humans. To simulate a DMD phenotype, we injected morpholino oligonucleotides into the yolk of zygote stage zebrafish embryos, causing a selective decrease in expression of the dystrophin protein and compromising muscle function and development. During the segmentation period of zebrafish development, we monitored the SHG signal from entire somite muscle compartments within the fish over time. This allowed us to monitor structural changes in the sarcomere, the muscular functional unit, over time without the use of exogenous dyes or transgenic fluorescent markers. Preliminary results show a marked delay in tissue development over time in the morpholino knockdown embryos in comparison to wild-type control fish. Additionally, morphant embryos had weaker muscle fibers that would tear over time as muscles started to become active, which was also characterized by endogenous SHGi. Since the structure and activity of the sarcomere determines the efficacy of muscle function, we assert that better early characterizations of the development of muscle tissue using SHGi as we have performed in this study will be integral in early diagnosis of a variety of neuromuscular disorders. Upon diagnosis of disease, SHGi can provide a minimally invasive overview of muscle tissue health in regions that are most affected as a means of monitoring the progression and benefit of treatment strategies for these disorders.

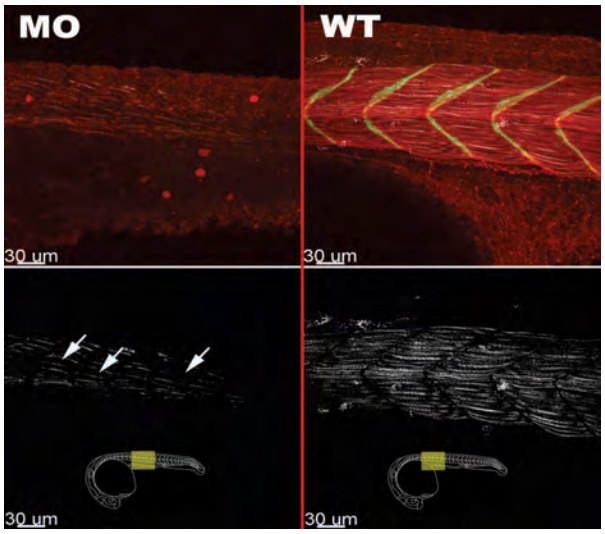


Figure 1: Morphant (MO) and wild-type (WT) embryos at ~24 hours post fertilization. MO embryo in column 1 has marked reduction in dystrophin protein at the sarcomere boundaries (green, top left box) in comparison to WT (green, top right box). SHG from muscle tissue is reduced due to a delay in development in the MO embryo (white, bottom left box), with gaps in muscle fibers (arrows). Red in upper boxes (phalloidin-Alexa546) labels cell compartments. Yellow boxes on inset zebrafish illustrations show-imaging locations within the fish.

44. Stimulation and control of neural function using millimeter and submillimeter waves

Peter H. Siegel, Scott E. Fraser, Victor Pikov[*]

As the application and commercial use of millimeter- and submillimeter-wavelength radiation becomes more widespread, there is a growing need to understand and quantify both the coupling mechanisms and the impact of this long wavelength energy on biological function. Independent of the health impact of high doses of radio frequency (RF) energy on full organisms, which has been extensively investigated, there exists the potential for more subtle effects, which can best be quantified in studies which examine real-time changes in cellular functions as RF energy is applied. In this program we are developing and employing instrumentation for the first real-time RF monitoring of RF-induced changes in cellular activity at absorbed power levels well below the existing safe exposure limits. Fluorescence microscopy imaging of immortalized epithelial and neuronal cells *in vitro* indicate increased cellular membrane permeability and nanoporation after short-term exposure to modest levels (10-50 mW/cm2) of RF power at 60 GHz. Sensitive patch clamp measurements on pyramidal neurons in cortical slices of neonatal rats show a dramatic increase in cellular membrane permeability resulting either in suppression or facilitation of neuronal activity during exposure to sub-μW/cm2 of RF power at 60 GHz-more than 3000 times below the US standards for maximum permissible RF exposure levels. These effects have been observed for the first time and represent a major paradigm shift in assessing the impact of this high frequency radiation on biological organisms. Non-invasive modulation of neuronal activity could prove useful in a variety of health applications from suppression of peripheral neuropathic pain to treatment of central neurological disorders.

[*]*Huntington Medical Research Institute, Pasadena, CA, USA*

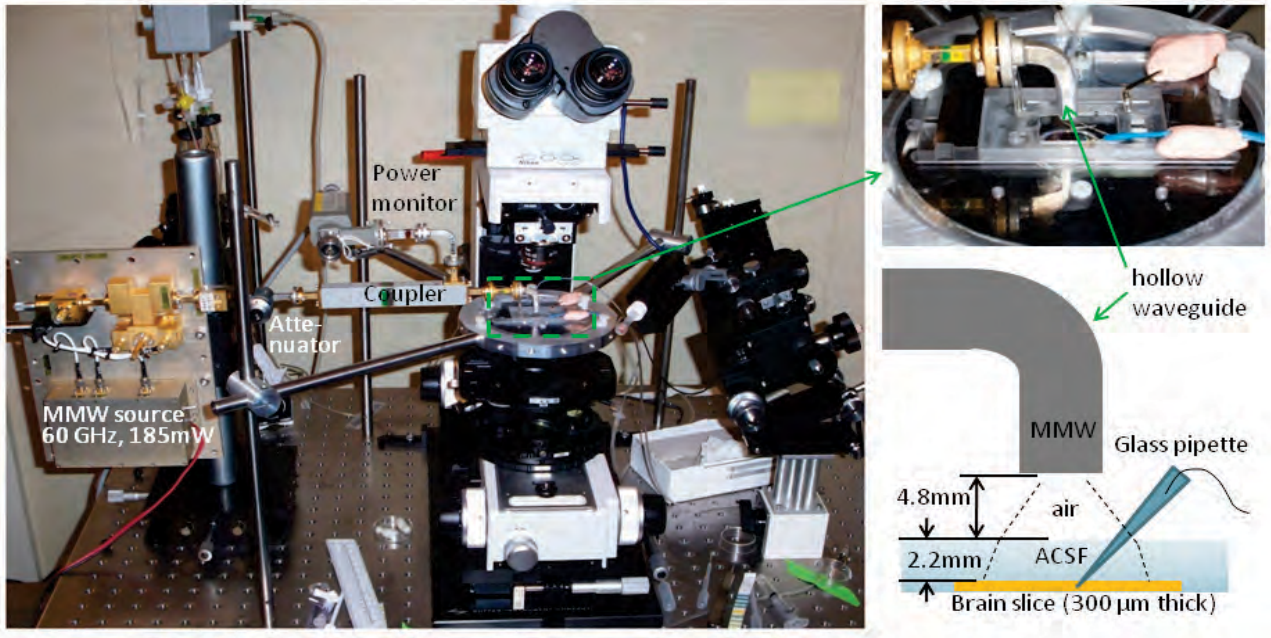


Fig. 1. Left. Photograph of patch clamp measurement set up with vertically coupled millimeter-wave power source (Impatt oscillator and WR15 output waveguide) for exposing cortical slices immersed in artificial cerebro-spinal fluid. Above right. Close up showing waveguide and tissue chamber. Below right. Schematic showing probe geometry and distances used for calculating the beam profile and power distribution at the tissue slice.

45. Ultra-high-Q microtoroid resonators for single cell proteomics

Judith Su, Scott E. Fraser

A single cell assay is needed to fundamentally understand protein function and expression; however, current detectors cannot sense the small amount of protein present in one cell that is typically on the order of zeptomoles. Recently, microtoroid resonators have been shown capable of achieving label-free single molecule detection. We demonstrate that a 50-fold improvement can be made in detection sensitivity than has previously been reported even with lower quality toroids. This corresponds to a 109 times improvement over the current industry gold standard. We are working on integrating our devices with microfluidics as a new and simple (fewer processing steps) means to achieve single cell proteomics. Currently we are working on early detection of cancer experiments (in collaboration with A. Raubitschek, City of Hope) and validating the binding data taken with the toroid by direct comparison with industry standard instrumentation, Biacore Surface Plasmon Resonance (in collaboration with A. Goldberg, J. Vielmetter, P. Bjorkman, and B. Stoltz). In addition we have developed a new way to sense the binding of molecules to the sensor that is far more robust than has been previously reported (in collaboration with K.J. Vahala).

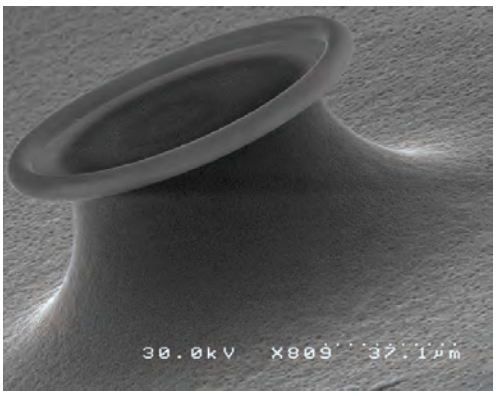


Figure 1. SEM image of an ultra-high-Q microtoroid resonator

46. Dynamic analysis of vascular morphogenesis using transgenic quail embryos

Yuki Sato[1], Greg Poynter[1], David Huss, Michael B. Filla[2], Andras Czirok[2], Brenda J. Rongish[2], Charles D. Little[2], Scott E. Fraser, Rusty Lansford

Background: One of the least understood and most central questions confronting biologists is how initially simple clusters or sheet-like cell collectives can assemble into highly complex three-dimensional functional tissues and organs. Due to the limits of oxygen diffusion, blood vessels are an essential and ubiquitous presence in all amniote tissues and organs. Vasculogenesis, the *de novo* self-assembly of endothelial cell (EC) precursors into endothelial tubes, is the first step in blood vessel formation. Static imaging and *in vitro* models are wholly inadequate to capture vascular pattern formation *in vivo*, because vasculogenesis not only involves dynamic changes of the endothelial cells and of the forming blood vessels, but the embryo is also changing size and shape.

Methodology/Principal Findings: We have generated Tie1 transgenic quail lines Tg(*tie1*:H2B-eYFP) that express H2B-eYFP in all of their endothelial cells that permit investigations into early embryonic vascular morphogenesis with unprecedented clarity and insight. By combining the power of molecular genetics with the elegance of dynamic imaging, we follow the precise patterning of endothelial cells in space and time. We show that during vasculogenesis within the vascular plexus, ECs move independently to form the rudiments of blood vessels, all while collectively moving with gastrulating tissues that flow toward the embryo midline. The aortae are a composite of somatic-derived ECs forming its dorsal regions and the splanchnic-derived ECs forming its ventral region. The ECs in the dorsal regions of the forming aortae exhibit variable mediolateral motions as they move rostrally; those in more ventral regions show significant lateral-to-medial movement as they course rostrally.

Conclusions/Significance: The present results offer a powerful approach to the major challenge of studying the relative role(s) of the mechanical, molecular, and cellular mechanisms of vascular development. In past studies, the advantages of the molecular genetic tools available in mouse were counterbalanced by the limited experimental accessibility needed for imaging and perturbation studies. Avian embryos provide the needed accessibility, but few genetic resources. The creation of transgenic quail with labeled endothelia builds upon the important roles that avian embryos have played in previous studies of vascular development.

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47. Mechanistic perspective of early vertebrate cardiogenesis

Jennifer Yang, Michael Liebling, Rusty Lansford Scott E. Fraser

Recent technological advances in dynamic confocal microscopy and four-dimensional reconstruction have shown that the embryonic heart tube in the developing zebrafish functions more as a dynamic suction pump than a peristaltic pump, disputing a theory cardiac physiologists had long held. Along with technological advances, recent research has shown the impact of flow in heart morphogenesis and valve development. As scientific methods and tools improve, previous conclusions of cardiogenesis need to be revised to incorporate new data at the molecular level.

The Japanese quail is an ideal system to study the pump mechanics of the four-chambered heart because it is a warm-blooded vertebrate with easily accessible embryos. All stages of the developing heart during looping and chamber formation are accessible *in ovo* and *ex ovo*, allowing for ease of manipulation of the embryo for dynamic imaging. Knowledge obtained from an avian four-chambered heart can then be applied to the less accessible mammalian and human hearts.

The mechanics of the developing embryonic heart are still poorly understood. Characterization of the interactions of the different layers of the heart will give us information on the contractile wave moving along the heart muscle, the offset of the contractile wave as it transverses the cardiac jelly to appear in the endocardium, and the mechanical importance of cardiac jelly. In order to characterize the heart, we used two independent transgenic lines of quail: Tg(tie1:H2B::eFYP) and Tg(pgk:H2B::mCherry). The Tie1 promoter localizes the eYFP signal to the nuclei of endothelial cells while the PGK promoter ubiquitously expresses mCherry in all cells. By crossing the two lines, we produced a novel transgenic with the myocardium labeled red and the endocardium doubly labeled yellow and red. Our results give a detailed fate map of heart morphogenesis, which begins to elucidate the mechanism of heart tube formation and provide new insight into cardiac looping.

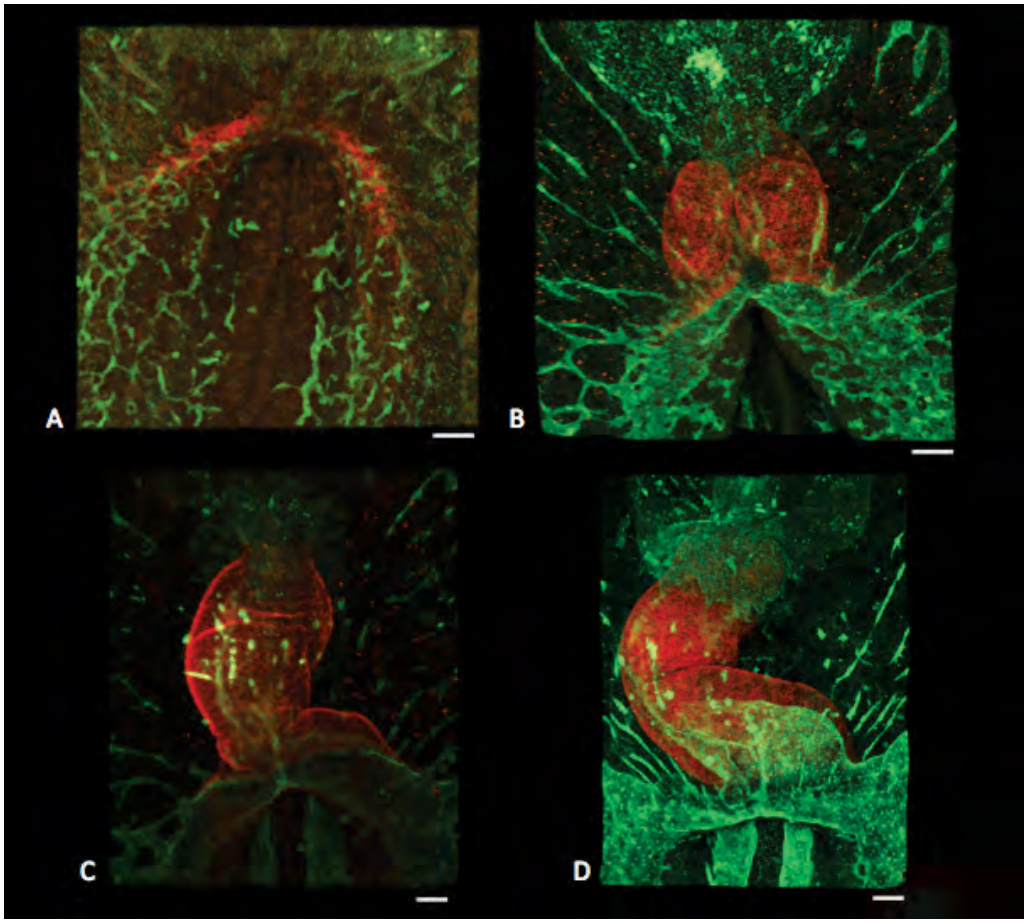


Figure: Wholemount immunostainings showing the heart at different stages of development. The red is the antibody MF20 that binds to the sarcomeres of the myosin heavy chain in the cardiac muscle. The green is the antibody QH1 that binds to endothelial cells. A) HH8, B) HH9, C) HH10, D) HH11. Scale bar is 100 μm for all images.

48. Imaging transcription factor dynamics in early mouse embryos

Nicolas Plachta, Shirley Pease[*]*, Scott E. Fraser, Periklis Pantazis*

A number of transcription factors have emerged as key proteins controlling the establishment of the first differentiated cell lineages of the mammalian embryo. It remains unclear, however, how these transcription factors segregate their activities among early blastomeres of the embryo, since many of these are initially expressed in all cells of the embryo. Our work focuses on studying how the dynamic behaviors of these proteins inside single cells of the embryo relate to the patterning of the first cell lineages. We use the mouse embryo as a model system. We culture these embryos from the 1-cell stage to the late blastocyst stage outside the uterus of the mother. During this developmental period (the pre-implantation period), all cells have similar developmental potential until the 8-cell stage, when the cells start differentiating into the first two lineages of the embryo (i.e., the pluripotent and the extra-embryonic lineage). By microinjecting RNAs directly into the 1-cell stage, we express transcription factors tagged with different fluorescent proteins and image the dynamics of these labeled molecules using time-lapse confocal imaging. We have found that the transcription factor Oct4, a key protein important for maintaining pluripotency in the embryo, as well as in cultured pluripotent stem cells, exhibits distinct dynamic behaviors among early blastomeres. Moreover, we found that distinct dynamics predict to which cell lineage the early blastomeres will be allocated at later developmental stages. These findings help towards better understanding of how early cell-to-cell variability can be established among apparently identical blastomers in the early embryo. While most studies have classically compared cells in the embryo by assessing the total levels of different proteins or the level of their corresponding mRNAs, our results indicate that early developmental heterogeneities can already be assessed even among morphologically identical blastomeres by differences in the dynamic behavior of a transcription factor. This type of finding also helps explain why, unlike other species, mammalian embryos show a high level of cellular plasticity during early development.

[*]*Genetically Engineered Mouse Services, California Institute of Technology*

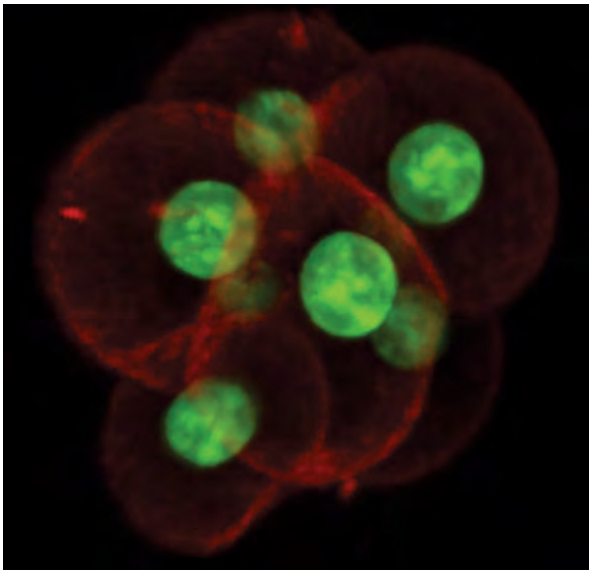


Figure legend: Cultured mouse embryo at the 8-cell stage injected with RNAs encoding for nuclear-targeted green fluorescent protein and for a membrane-targeted red fluorescent protein.

49. Redefining the olfactory necklace in terms of the Grueneberg Ganglion

David S. Koos, Scott E. Fraser

The Grueneberg Ganglion (GG) has recently been recognized as an olfactory organ in most mammals, distinctly located at the tip of the nose where it is ideally situated to be the first olfactory subsystem to interogate inhaled chemicals. In the mouse, the GG projects to the olfactory necklace domain, an unusual sub-region of the olfactory bulb containing interconnected glomeruli that appear like beads on a string encircling the entire caudal olfactory bulb. The olfactory necklace domain also receives afferent input from another olfactory subsystem comprised of GC-D expressing neurons. A major mystery is how these two distinct populations interact and contribute to those unusual necklace glomeruli structures, and whether or not individual necklace glomeruli are innervated by both populations or are exclusively innervated by one or the other. To address this fundamental question, we are microscopically dissecting the olfactory necklace glomeruli. By combining state of the art imaging technologies with genetic labeling, inducible axon tract tracing and whole-mount immunolabeling, we generate large-scale, high-resolution, extended-volume datasets that represent the olfactory necklace axons and their cognate glomeruli. 3D reconstructions of these data allow us to explore the physical relationships between the two different classes of necklace axons, and demonstrate the amount of overlapping glomerular innervation (or lack thereof) among these populations. Our preliminary results indicate that the olfactory necklace domain contains multiple, genetically-defined axon necklaces of glomeruli. There appears to be no overlapping glomerular innervation between these populations, even though these necklaces of interconnected glomeruli occupy the same region of the olfactory bulb. This suggests that the GG necklace and the GC-D necklace have their own exclusive glomeruli. This high-resolution connectivity map of the olfactory necklace glomeruli lays the foundation for functional analysis of the olfactory necklace domain's response to odorants and its relation to animal behavior.

50. Measurements of intracellular calcium in isolated Grueneberg ganglion cells

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

Transient elevations in cytosolic calcium concentration correlate with neuronal electrical activity in many types of neurons. Primary mouse olfactory neurons exhibit calcium bursts in response to stimulation with specific odorants. We have developed a technique to measure intracellular calcium levels in an unusual set of olfactory neurons, the Grueneberg ganglion (GG), whose specific stimulatory odorants remain unknown. This technique centers upon the dissociation and isolation of GG neurons apart from other cell types in the far-anterior mouse nasal vestibule, their identification using their selective expression of mCherry by the olfactory marker protein promoter, and their subsequent loading with the calcium-responsive green fluorescent dye fluo-4. GG neurons adhere to flat glass coverslips and remain viable for calcium imaging for up to 8 hours after their dissociation. Perfusion of the imaging chamber with a hyperkalemic buffer induced a sustained rise in fluo-4 fluorescence ($\Delta F/F$ as high as 1.5) that could be reversed upon washout. Our technique enables the simultaneous tracking of several neurons in the same focal plane in each experiment and facilitates the localization of calcium bursts to GG neurons distinct from neighboring cell types in the tightly-packed mouse nasal vestibule, factors that challenge the calcium imaging technique in other (i.e., slice culture) preparations of the GG. We anticipate that our technique will aid identification of GG ligands by allowing the assay of GG cytoslic calcium levels in response to perfusion with various potential odorants. Ultimately, this will help define the still-mysterious function of GG neurons in the context of the mouse olfactory sense.

51. *In vivo* distinction between plasma membrane and near-membrane organelles using variable angle total internal reflection fluorescence microscopy (VA-TIRFM)

Larry Wade, Henry Lester, Scott E. Fraser

We have imaged cellular membranes in near isolation from cell organelles using variable-angle TIRFM. Further, we have captured 'z-sectioned' pictures of cells only 250 nm thick. This provides 3D information similar to that gained by confocal microscopes but with at least an order-of-magnitude greater z resolution. Key experimental procedures include: (1) a controllable excitation laser micropositioner in a standard through-the-lens TIRF illuminator; (2) a custom culture dish for re-use of expensive high index of refraction cover slips. Images are acquired at several penetration depths by varying the excitation laser illumination angles. At the shallowest penetration depth (~46 nm) just the membrane and a few internal puncta are imaged. As the penetration depth is increased up to 250 nm organelles near the membrane, such as the ER, are imaged, as well. By subtracting the 'shallower' image in a sequence from the next 'thicker' image a stack of images is generated of ~constant thickness at increasing distance from the coverslip. We employ this method to distinguish membrane-localized fluorophores (ER Tracker, alpha4beta2 nicotinic acetylcholine receptors) at the plasma membrane (PM) from those in near-PM endoplasmic reticulum, on a z-axis distance scale of ~45 to ~250 nm in N2a cells. In doing so we observe occasional smooth ER structures that cannot be resolved as being distinct from the membrane. We anticipate that the ability to resolve cellular membranes and nearby cellular organelles, and to optically interrogate them independently, will prove valuable to studying trafficking and metabolic pathways involved with membrane bound and embedded proteins.

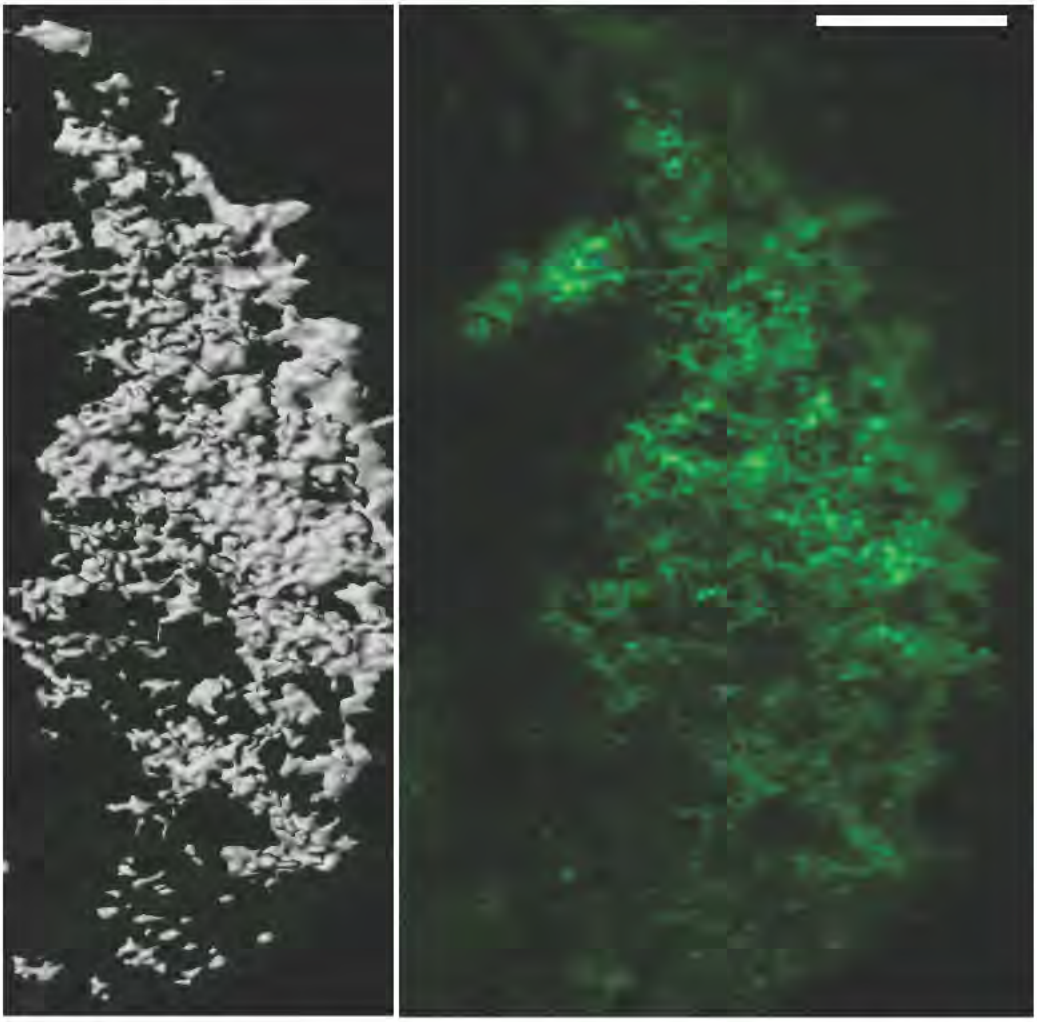


Figure 1. A 3D image provides information about cell structures within 250 nm of the membrane with high fidelity. These images show the view from the inside looking out of a 3D reconstruction that was made from differenced images in using the identical techniques that generated figure 9. On the right, a 240 nm penetration depth TIRFM image is shown of a N2a cell that was transfected with α4-eGFP β2-365AAQA368L349M nAChRs. On the left is a thresholded surface image (497 counts/pixel after background subtraction and normalization) of the same cell. The scale bar at the upper right is 12 μm long.

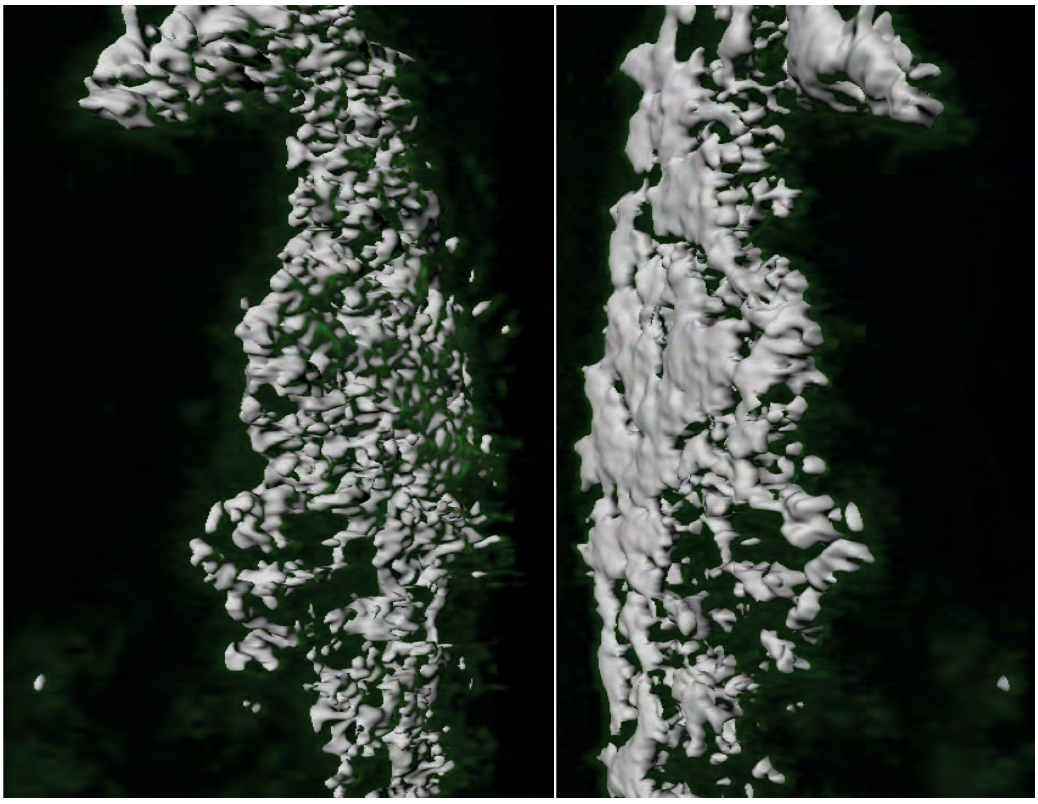


Figure 2. Intricate detail is visible indicating that there are many places where parts of the smooth ER are proximate to the membrane such that they cannot be resolved as independent structures. This is the same 3D reconstruction seen in Figures 10 and 11, of a N2a cell transfected with α4-eGFP β2-365AAQA368L349M nAChRs. The image on the left is further rotated so that the left hand edge seen in Figure 10 is nearly perpendicular to the page. The direction of rotation is continued so that the view shows the reconstructed 3D cell image from the membrane side looking in.

52. CNV imaging in a laser-induced mouse model using swept source OCT at 1050 nm

Reza Motaghian, David Koos, Shikun He[*], David Hinton[*], Scott E. Fraser

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss and legal blindness in Americans aged 60 and older. Choroidal neovascularization (CNV) is a serious complication associated with "wet" types of AMD that affect` (10-15%) of all AMD cases. The occurrence of CNV is particularly devastating because it can cause rapid and severe vision loss within weeks. CNV is characterized by a neovascular submacular membrane that is formed as newly-formed fragile blood vessels originating from the choriocapillaris infiltrate through Bruch's membrane and leak. Thus, there is an unmet need to identify the earliest transitions from dry AMD to wet AMD for the early diagnosis and efficient preventative treatment of CNV before vision loss occurs. Although fluorescein angiography (FA) is a well-established objective diagnostic test of CNV, it is somewhat costly, invasive, and time consuming. Moreover, because of a lack of adequate contrast and depth information, FA may have limited efficacy for detecting the earliest, small neovascular lesions that are exhibiting little or no leakage. Optical coherence tomography (OCT) is an interferometric imaging technique that is able to provide cross-sectional reflectance and phase images. OCT has been employed as an emerging technology in ophthalmology and Doppler OCT has been applied for imaging blood flow in the retina. We have recently demonstrated phase contrast spectral domain OCT for retinal/choroidal visualization. A swept source OCT at 1050 nm is demonstrated to visualize mouse retinal/choroidal microvasculature and vessels *in vivo*. We show that OCT contrast image highlights the region of abnormality and motion in the retina, as well as the choroid. In order to test this technique for visualizing the leaking fluids in CNV lesion, we performed SS-OCT in a mouse laser-induced model of CNV. We were able to visualize the area of leakage in CNV lesion using OCT method. Thus, a SS-OCT system at 1050 nm offers assays associated with the earliest transition from dry AMD to wet AMD and provide a reliable measure for treatments and therapies, as it may be capable of identifying abnormal vessels and leaking fluids with a high sensitivity before significant visual loss ensues.

[*]*Departments of Pathology and Ophthalmology, Keck School of Medicine, Arnold and Mabel Beckman Macular Research Center, Doheny Eye Institute, Los Angeles, California*

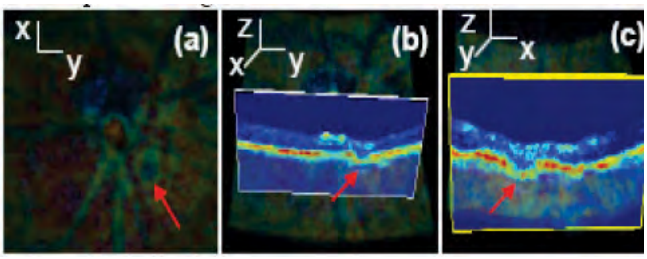
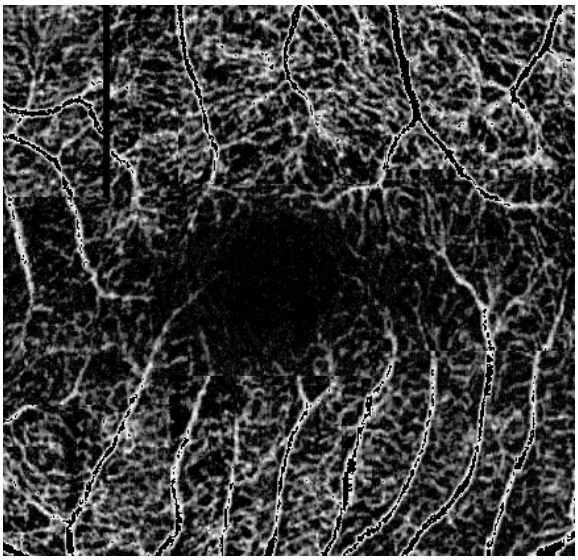


Figure: 3D intensity contrast image of an *in vivo* mouse laser-induced model of DNV retina from different views.

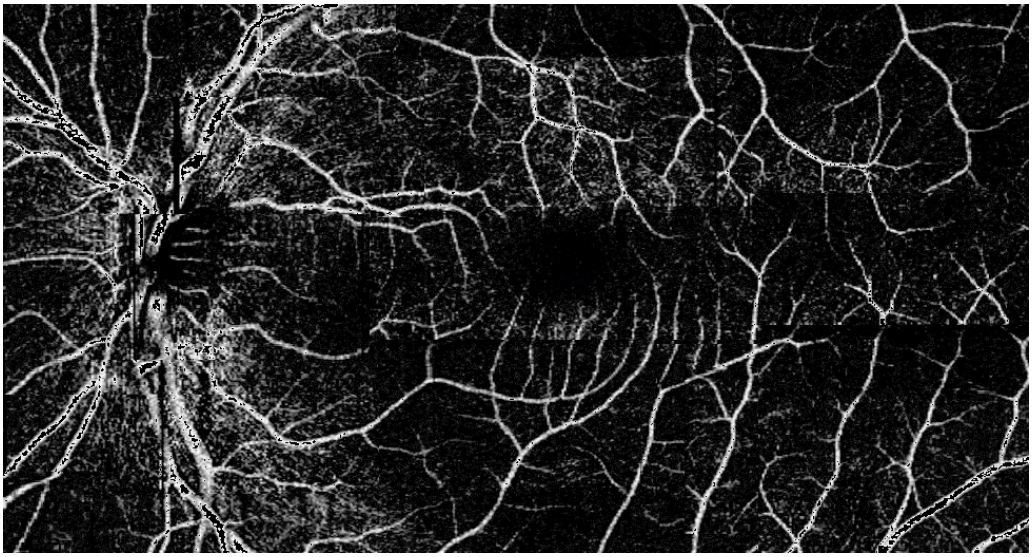
53. Retinal vascular imaging using phase contrast optical coherence tomography

Jeff Fingler, Scott E. Fraser

Non-invasive retinal vascular imaging is important for early screening of vascular diseases in the eye, such as age-related macular degeneration (AMD) and diabetic retinopathy. Early detection of diseases is key to developing new treatment options and improving the effectiveness of current treatments. Phase contrast optical coherence tomography (PC-OCT) has been developed to three-dimensionally image the vasculature within the retina non-invasively with an unprecedented level of microvascular visualization. Composite imaging techniques have been applied to align and stitch together multiple data sets to achieve large vascular images while maintaining short retinal imaging acquisition times. This technique has been applied towards large vascular scans extending over the optic nerve head and the fovea, demonstrating visualization of the major vascular features, but also applied towards smaller vascular scans to visualize the finer microvascular features.



High resolution PC-OCT composite image of retinal micro-vasculature over an area of approximately 2mm x 2mm.



PC-OCT composite image of retinal vasculature over an area of approximately 10mm x 5mm.

54. Axonal injury and myelin loss in glutaric academia type 1 (GA-1) mouse model

Jelena Lazovic, Natalia Malkova, Russell E. Jacobs

Glutaric acidemia type I (GA-1) is an inborn error of lysine, hydroxylysine and tryptophan catabolism, with deficient glutaryl-CoA dehydrogenase (Gcdh) activity. The features of leukoencephalopathy are frequently found upon MRI [1,2]. We hypothesized that constant production of glutaric acid within the brain will lead to neurotoxicity, axonal injury and impaired myelin maintenance.

Gcdh-/- mice and wild-type (WT) controls (N=8 Gcdh-/-, N=6 WT mice) were imaged prior to, and after 10 months on the lysine-enriched diet (4.8% total lysine in the diet). We measured T2-values to assess axonal damage, and magnetization transfer ratio (MTR) to determine myelin loss. The behavioral tests included acoustic startle response and open filed tests. Statistical analysis was performed using one-way ANOVA, followed by a Holm-Sidak post-hoc test.

Following 10-months of lysine-enriched diet significantly increased T2-values (ρ<0.05) were present in the striatum of Gcdh-/- mice (41.2 ± 3.05 ms) *vs.* WT (30.8 ± 2.7 ms), **Figure 1 A, B**. Hypointense lesions were found in the thalamus of Gcdh-/- mouse exposed to lysine-enriched diet, **Figure 1 C, D**. Reduced MTR were found throughout the brain of Gcdh-/- mice, and significantly reduced MTR (ρ<0.05) were found in the striatum of Gcdh-/- (0.12 ± 0.03) *vs.* WT (0.21 ± 0.03) mice on lysine-enriched diet, **Figure 1 E**. Behavioral testing revealed significantly decreased (ρ<0.05) acoustic startle response in Gcdh-/- mice compared to age matched Gcdh-/- maintained on the normal diet and WT on both lysine-enriched and normal diet, **Figure 2**. The open field test detected reduced locomotor activity and anxiety-like behavior only in Gcdh-/- exposed to lysine-enriched diet for 10 months. Thalamic lesions found in our study, open up the possibility that myelin degradation is secondary to axonal damage. While the exact mechanism of myelin degradation in GA-1 remains to be clarified, presented data indicate a strong correlation between long-term lysine exposure and neuropathology in Gcdh-/- mice, arguing for life-long dietary restrictions for GA-1 patients.

Figure 1. A, B Representative T2-WI and T2-maps for WT and Gcdh-/- mice both on lysine-enriched diet for 10 months. Notice increased signal on T2-WI and elevated T2-values in the striatum of Gcdh-/- mouse.

Figure 1. C Representative T2-WI at the level of thalamus (same mice), and **D** matching high-resolution gradient recalled echo images. Red arrowheads are pointing to the bilateral thalamic lesions in Gcdh-/- mouse. Note additional lesions in cerebellum and pons in Gcdh-/- mice on high lysine diet for 10 months.

Figure 1. E Calculated MTR images (same slice and mice as in A, B), and **F** corresponding histology and staining for neurofilaments (N52, green) and astrocytes (GFAP, red). Scale bar is 37.5 μm. Color scale shows different T2-values and MTR percentages.

Figure 1 F. A specialized staining for neurofilaments (N52) revealed decreased number of axons, part of the fiber bundles passing through the striatum (striatal white matter patches).

Figure 2. Magnitude of acoustic startle response, measured as induced voltage. Significantly reduced startle response in Gcdh-/- mice exposed to lysine-enriched diet compared to age-matched Gcdh-/- on normal diet and WT on lysine-enriched diet (* p<0.05, one-way ANOVA).

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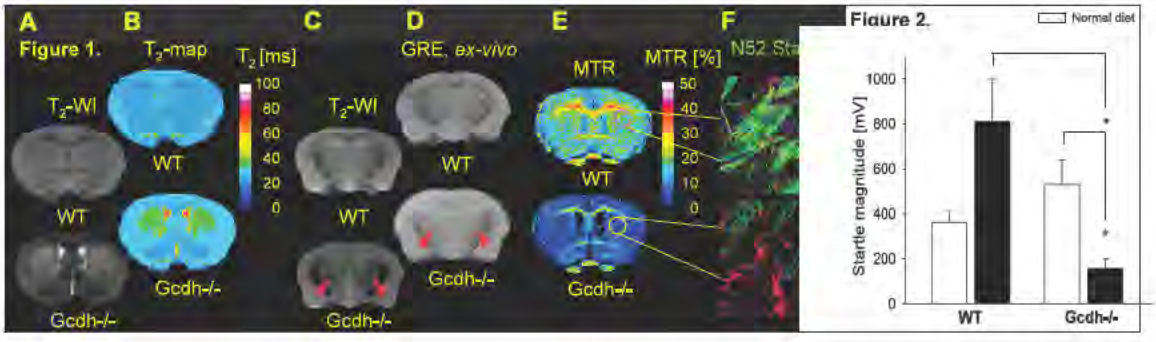
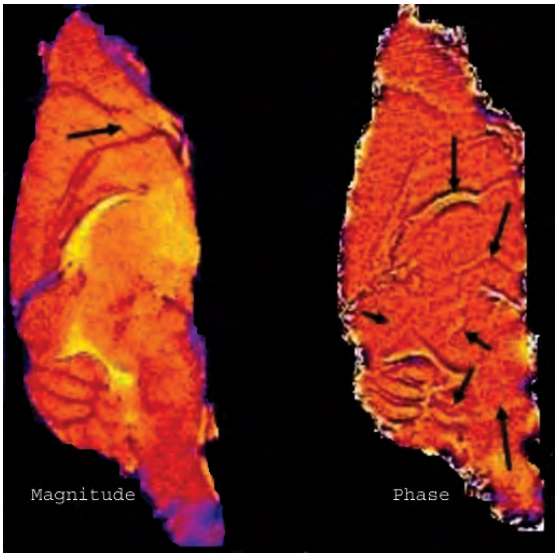


55. Weighted imaging in the rodent brain at high field

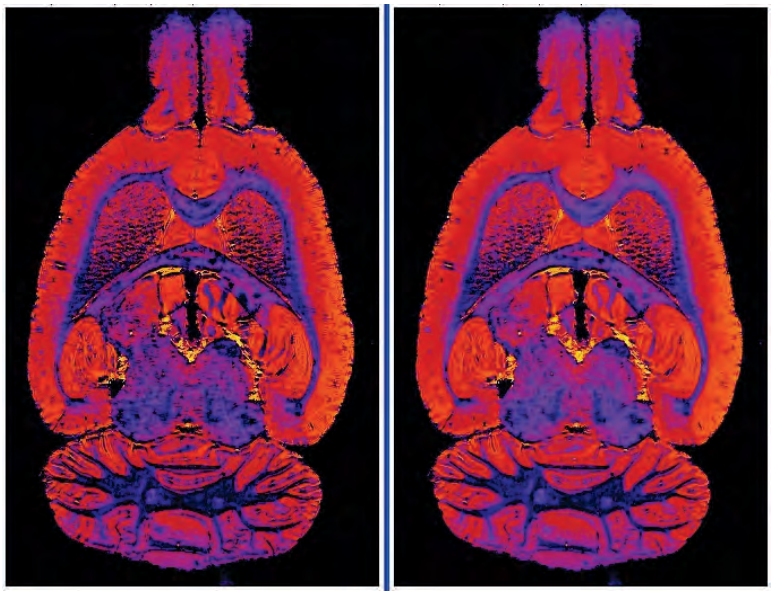
Benoit Boulat, Russell E. Jacobs

Magnetic susceptibility of a material characterizes its response to an external magnetic field. This offers another form of contrast enhancement in MRI that may be indicative of blood vessels and clot locations, subtle difference in tissue types or iron-laden tissues. So called "phase" images are used in MRI to interpret susceptibility contrasts. Phase images can be analyzed directly or utilized to obtain Susceptibility Weighted Images (SWI) in which some of the phase contrast is superimposed onto the magnitude image by multiplying with one another with various degrees the intensities of the phase image and of the magnitude image.



Representative magnitude (left) and phase (right) images recorded in a live mouse brain. Arrows are indicative of differences in contrast obtained between the two modalities.

To derive both images a single gradient echo experiment was utilized, echo time TE = 10ms, recovery time TR = 1 second, flip angle = 60 degree. The in plane resolution is 78 micrometer, slice thickness is 400 micrometer. The phase image is obtained by mapping the original phase values PH in the interval [0,1] to 1 and those in the interval [-1,0[, to the interval 4.57 + PH.



SWI in a fixed rat brain. Negative phases are mapped to the interval [0,1], while positive phases are set to exactly one. The values thus obtained, are then multiplied, five times (right) or ten times (left) to the corresponding voxel intensity in the magnitude image. A gradient echo modality was used (TE = 10 millisecond, flip angle =10 degree, TR = 50 millisecond) to obtain both magnitude and the phase images. Resolution is 50 microns in the plane, slice thickness is 50 micron. The brain was soaked in Prohance prior to imaging.

56. Development of simultaneous PET/MR technology for small animal imaging

Thomas Ng[1], Hargun Sohi[1], Daniel Procissi[1], Yibao Wu[2], James Bading[3], Ryan Park[4], Andrey Demyanenko[1], Simon R. Cherry[2], Russell E. Jacobs[1]

The study of biological processes *in vivo* is vital for their proper elucidation. Positron Emission Tomography (PET) and Magnetic Resonance (MR) imaging are two non-invasive imaging modalities that offer complementary functional and anatomic information [1]. While retrospective fusion of individual PET and MR datasets has shown useful correlations of the multi-modal datasets [2], simultaneous acquisition of PET/MR images allows time sensitive biology to be studied from multiple perspectives without difficult and complex spatial co-registration of the multi-modal data. Building upon our previous work of developing the first MR-compatible PET insert instrumentation for small animal imaging [3,4], we are developing and characterizing the integrated PET/MR system for use in robust *in vivo* studies. A simple yet accurate spatial alignment and processing pipeline was developed to ensure good registration of the PET and MR images [5]. The PET and MR electronics operating systems were integrated to allow synchronous dynamic imaging. This combined with parallel computing algorithms enable time-sensitive *in vivo* studies to be performed in an efficient manner. For example, this integrated system setup allowed us to compare PET and MR metabolic signatures of a tumor cell mass in real time. Rapid processing and analysis of fluorodeoxyglucose (FDG) PET/ MR image datasets guided the acquisition of magnetic resonance spectra from differential regions of glucose uptake. High regions of glucose uptake correlated with those of high choline signal, which suggests regions of high cellular activity.

Ongoing work aims to improve the combined PET/MR technology. We are harnessing recent advancements in solid-state photodiode technology and mechanical engineering designs to improve the efficiency of the detector hardware. Studies characterizing the current integrated system and novel detector setups will be used to guide the design of next generation of PET/ MR integrated systems [6]. To maximize the flexibility and power of this system, we are also developing protocols to enhance the range of complementary information that can be obtained using the PET/MR, through developing novel imaging sequences and techniques coupled to the use of interesting contrast agents.

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57. Understanding kinetics and dynamics of cancer nano-therapies *in vivo* via imaging

Thomas Ng, Hargun Sohi, Daniel Procissi, Bita Alaghebandan, David Wert[*], James Bading[*], Andrew A. Raubitschek[*], Russell E. Jacobs

Nanoparticle [1] and antibody [2] cancer therapies are promising because of their ability to target within a specific site in the body, the tumor mass. Increased uptake of the drug within the tumor site reduces systemic toxicity while concentrating localized response. It is likely that both classes of therapies have specific therapeutic dosage windows that maximize their efficacy. Current methods to understand and evaluate the efficacy of these therapies include tumor size measurements, *in vitro* biochemical assays and intra-vital microscopy. PET and MR imaging complement these studies, allowing longitudinal studies of drug uptake and response within the tumor environment. Moreover, these techniques are clinically translatable. We have developed PET and MR imaging techniques to understand the kinetics of antibody uptake within the context of a heterogeneous tumor. For example, Figure 1 shows a changing pattern of tumor localizing antibodies over the period of 2 days, suggesting a heterogeneous tumor uptake of the antibody over time and space. This correlates with previous *ex vivo* and *in vitro* studies showing heterogeneous tumor uptake of antibodies [3] and highlights the utility of the simultaneous PET/MR technology. Apart from this, we have explored PET/MR methods to follow the response of tumors to nano-particle treatments. By examining the diffusion status of the tumor with MR, it is possible to determine the cellular density of the tumor mass [4]. Over the course of a week, we demonstrated that diffusion MR was sensitive to changes in tumors treated with a nano-particle chemotherapy [5.6] compared to controls, which correlated with anticipated size changes (Figure 2). Furthermore, heterogeneous patterns of response were observed within tumors *in vivo*, a finding not possible with traditional assays.

Ongoing work aims to further explore how the tumor bio-distribution of these classes of drugs affects tumor response *in-vivo*. Longitudinal *in vivo* and *ex vivo* assays of tumor histology will allow us to understand the efficacy of such theranostics, feeding back to guide better drug design, while providing a set of tools that can be used clinically to tailor personalized treatment regimens.

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Figure 1:

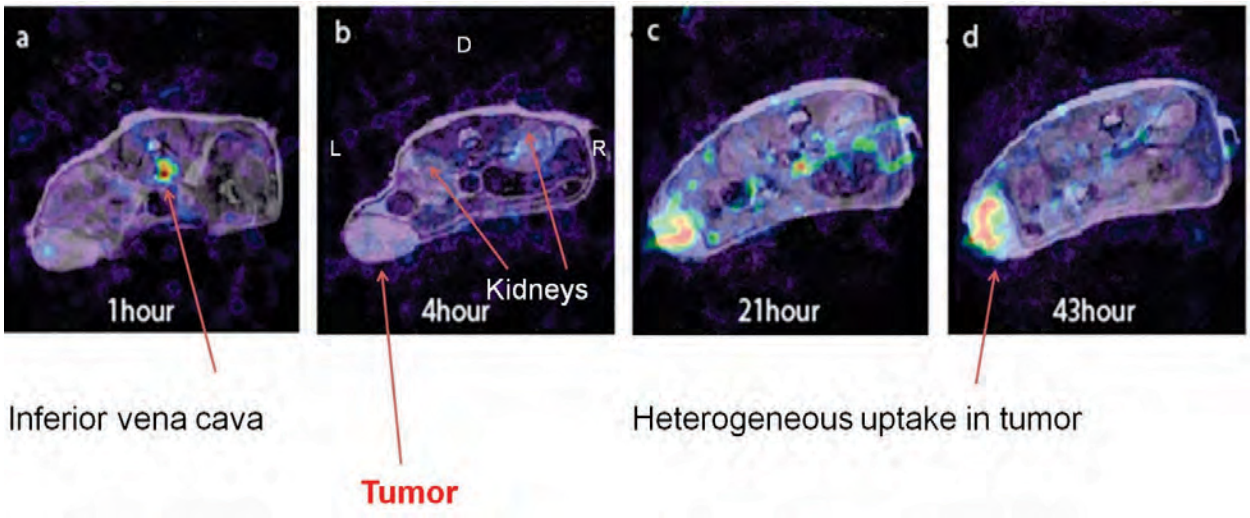
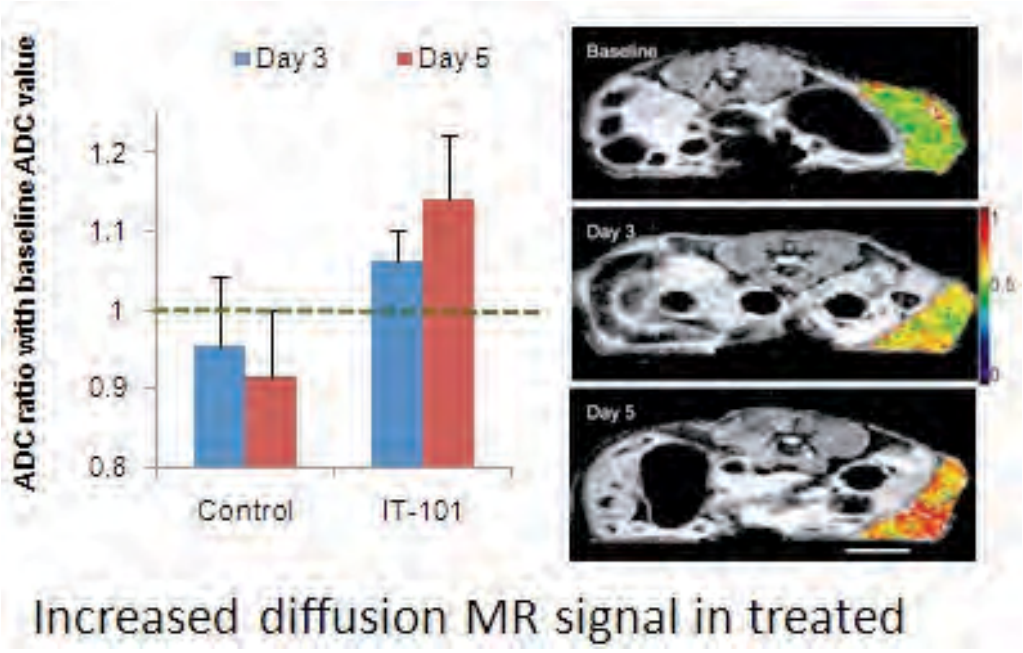


Figure 2:

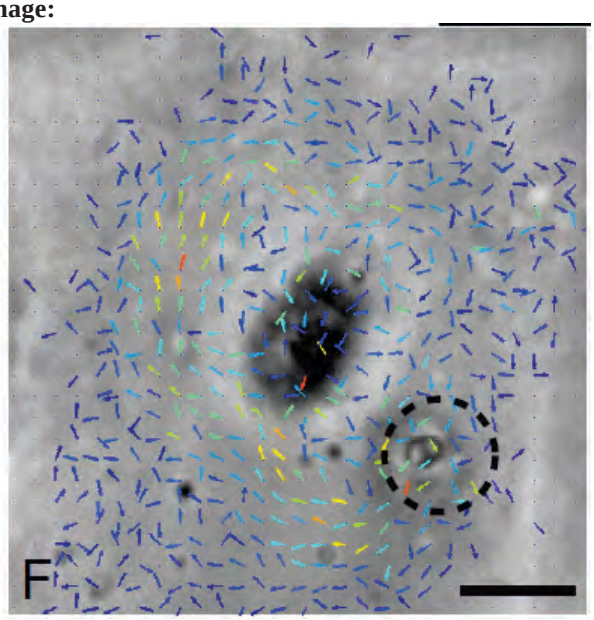


58. Hydrodynamics of otolith formation during teleost inner ear development

David Wu, Jonathan Freund, Scott E. Fraser, Julien Vermot

Biomineralization is the process by which metazoans form hard minerals. In vertebrates, mineralization occurs in numerous developing structures such as teeth, bones, carapaces, and otoliths. Otoliths, which are connected to stereociliary bundles in the inner ear, serve as inertial sensors for balance. In teleostei, otolith aggregation is critically dependant on flow forces generated by beating cilia; however, the mechanism by which flow controls otolith formation remains unclear. Here, we have developed a non-invasive flow probe using optical tweezers, and a viscous flow model in order to demonstrate how the observed hydrodynamics influence otolith assembly. We show that rotational flow in the core of the cilia-driven vortex suppresses precursor agglomeration and that the velocity field correlates with the shape of the otolith. An implication of this hydrodynamic effect is that otolith self-assembly is mediated by a balance between Brownian motion and cilia-driven flow. More generally, this flow feature highlights an alternative biological strategy for controlling particle localization in solution.

Image:



Caption: Flow around an otolith in the inner ear of a 19 hpf zebrafish as visualized by particle image velocimetry.

59. Synthetic *cis* Regulatory Modules

Roee Amit, Hernan Garcia, Rob Phillips, Frances Arnold, Scott E. Fraser

We would like to introduce a new paradigm for synthetic biology – Synthetic *cis* Regulatory Modules (SCRM). Namely, rather than design circuits, design and implement non-protein coding regions of DNA that regulate gene-expression in a precise 5-D pattern (time, space, and intensity). In nature, CRMs are the large non-coding genomic regions that occupy broad segments of the genome (sometimes tens of kb), and are implicated in the regulation of many genes. The regulation is due to a handful of transcription factors (typically 5-10), DNA binding proteins, and chromatin remodeling complexes that bind at several (typically clustered) binding sites each. Recently, detailed *cis* regulatory analysis carried out on several developmentally important genes in fly and sea-urchin model organisms has provided evidence that regulatory function carried out by CRMs are akin to complex computational operations.

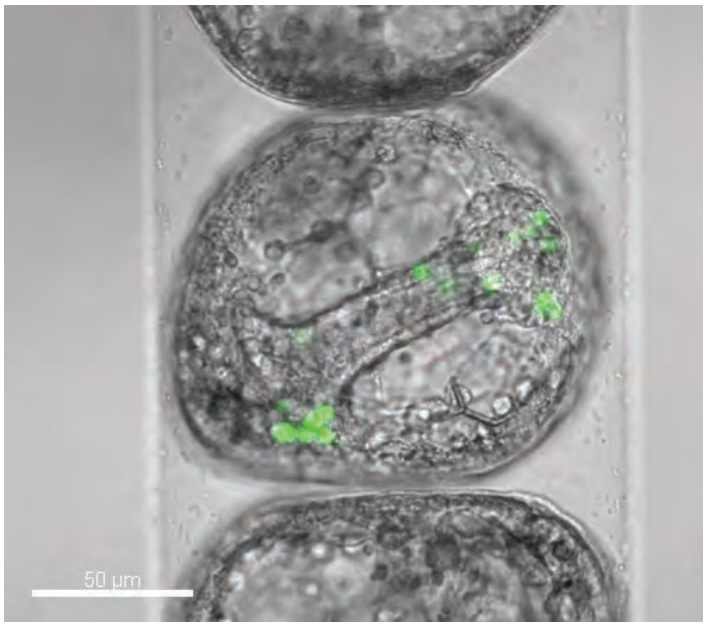
In order to address this question, my goal is to construct synthetic CRMs (SCRM) *de novo* with binding sites for commonly used transcription factors that will show emergent computational behavior. In order to control the number of variables, I chose to use a bacterial system consisting of a minimally active promoter with the poised alternative sigma factor σ54, and an associated upstream minimal enhancer. The SCRM sequences designed thus far, are divided into two classes: 1-cassettes that alter the spacing between the enhancer and minimal promoter; and 2-cassettes of either TetR or TraR binding sites added to the enhancer.

Our results divide into three levels of increasing complexity. First, we measured reporter expression as a function of the length separating the minimal promoter and enhancer and found that expression levels depend on both DNA helicity and probability of DNA looping. Second, we introduced a single binding site for TetR 22bp upstream of the activator-binding site, and found that the looping dependence induces a repression function which has two distinct states: one fully repressed and one only partially inhibited. Finally, we constructed multiple-binding site cassettes of either TetR or TraR, which exhibited a step like response of reporter expression levels to an input of variable amounts of externally supplied ligand (aTc or OOHl respectively). Thus, showing the potential to convert a chemical gradient input to an output step function. In conclusion, our simple synthetic enhancer system was able to recapitulate complex regulatory behavior that has been previously observed for natural CRMs.

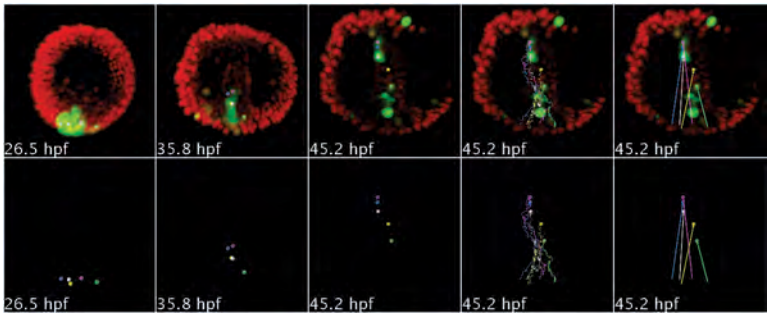
60. Developmental dynamics of the sea urchin embryo

Mat E. 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 make them ideal for study by light microscopy. We have recently developed a technique for multi-hour time-lapse imaging of living sea urchin embryos, revealing details of their development inaccessible to traditional techniques. For example, by tracking the migration of each cell in a single embryo over time, we are investigating the roles of specific genes involved in gastrulation. Traditionally, studies of dynamic gene expression have been performed by combining snapshots of different embryos collected at different developmental times. By comparing expression of a gene in one embryo to expression of the gene in a different embryo at a different developmental time, conclusions have been drawn about the dynamic expression of that gene. Such conclusions, however, are necessarily limited by the fact that, although different embryos share stereotypical cell types, a *different* embryo is viewed at each point in time, making direct comparisons between embryos difficult. Our technique removes this limitation by examining dynamic gene expression in a single embryo over time. Through such live imaging, we have – for the first time – directly observed the cell rearrangements that occur during gastrulation, and have begun to gain insight into the regulatory mechanisms that drive morphogenesis.



Live late-gastrula-stage sea urchin embryos. Expression of the transcription factor *foxa* is shown in nuclei of the foregut and hindgut, via injection of a *dendra2-foxa* fusion BAC.



Time-course of *foxa* BAC expression during gastrulation. The paths of five *foxa*-expressing cells were tracked, revealing their local rearrangement during archenteron formation.

61. Cytoskeletal polarity mediates localized induction of *Ciona* heart precursor lineage

James Cooley, Stacia Ilchena, Sarah Sweeney, Scott E. Fraser, Brad Davidson

Asymmetric cell division is a fundamental mechanism in developmental biology by which a single fertilized cell can develop into a multi-cellular organism. Previous studies have shown that asymmetric divisions are typically caused by a shift in spindle orientation and position. In the tunicate *Ciona intestinalis* one such division is required to establish the heart precursor cells. Each of the four cells which make up the B7.5 lineage at embryonic stage 12 (founder cells) divide asymmetrically at stage 15 to produce a large tail muscle cell and a smaller heart precursor cell. Previous research has shown that this asymmetric division requires a non-polarized, FGF signal from the adjacent mesenchyme, which results in uniform FGF receptor occupancy on the B7.5 cells. This causes a localized change in cytoskeletal dynamics, resulting in an asymmetric division of the B7.5 lineage.

Targeted expression of a dominant-negative form of the FGF receptor in the founder cell leads to symmetric division. Also, manipulation of Cdc42 and inhibition of MAPK signaling result in symmetric founder cell division. To better understand how these three molecules interact and their direct effect on division symmetry, we are investigating spindle dynamics within the founder cell. We are using live fluorescent microscopy to analyze microtubule dynamics as they relate to spindle orientation and shifts. In addition, we are using molecular cloning techniques to identify target candidate molecules that might also be involved in this pathway. High resolution analysis of founder cell spindle dynamics will determine how FGF signaling and cytoskeletal polarization regulate the asymmetry of size and fate. Live imaging will result in a further understanding of the interactions between actin and microtubule cytoskeletons. The question of whether these cytoskeletal interactions influence localized signal transduction or whether signaling impacts these interactions has yet to be addressed.

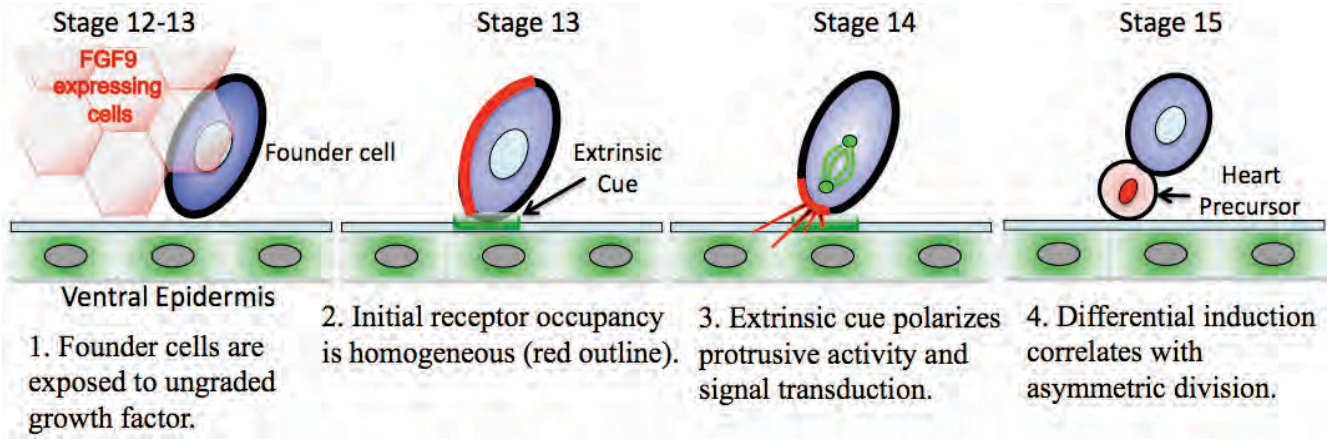


Figure 1. Model for differential specification of the heart precursor lineage
***Image and caption created by Brad Davidson for submitted paper: "Cytoskeletal polarity mediates localized induction of the heart precursor lineage," James Cooley, Stacia Ilchena, Sarah Sweeney, Scott E. Fraser, and Brad Davidson.

62. Endoderm morphogenesis impacts heart progenitor convergence in *Ciona*

Katerina Ragkousi, Sarah Sweeney, Scott E. Fraser, Brad Davidson

The *Ciona intestinalis* heart develops from two bilateral sets of myocardial precursors that migrate and converge at the ventral midline of the developing embryo. The bilateral fusion of *Ciona* heart progenitors is strikingly similar to the fusion of vertebrate myocardial precursors. Proper endoderm morphogenesis is required for convergence of these bilateral heart progenitors, but previous vertebrate studies have yet to resolve the role of the endoderm in heart precursor convergence and studies that directly examine the molecular and cellular mechanisms of endoderm/heart precursor interactions are very limited. Our research will determine the precise cellular mechanisms underlying *Ciona* endoderm morphogenesis and how this impacts neighboring developing tissue, in particular the heart precursor lineage. In vertebrate embryos, disruption of genes involved in the regulation of endoderm specification or morphogenesis consistently leads to cardia bifida. Functional analysis of *Ciona* endoderm morphogenesis reveals that GATAa is important for proper endoderm morphogenesis and if disrupted, affects heart precursor midline convergence. High resolution morphogenetic analysis of the developing endoderm indicates that heart precursors converge when in contact with endodermal hinge-point cells. These hinge-point cells are normally positioned symmetrically near the site of heart precursor convergence. However, when endodermal GATA function is disrupted, the heart precursors appear to associate with mislocalized hinge-point cells and fail to converge. This suggests that the conserved effect of endoderm morphogenesis on heart precursor convergence can be attributed to precise cellular interactions. To better understand the dynamic interactions between the forming endoderm and adjacent heart precursors, we are using live fluorescent microscopy to track the cellular interactions between discrete regions of the forming endoderm and the heart progenitors. We are particularly interested in how hinge-point cell position and morphology impacts neighboring endoderm and heart precursors. Live imaging analysis will determine how endoderm morphogenesis influences heart precursor convergence. Previous studies have examined the molecular basis of epithelial morphogenesis, but how changes in one tissue impact the morphogenesis and specification of adjacent lineages has yet to be addressed.

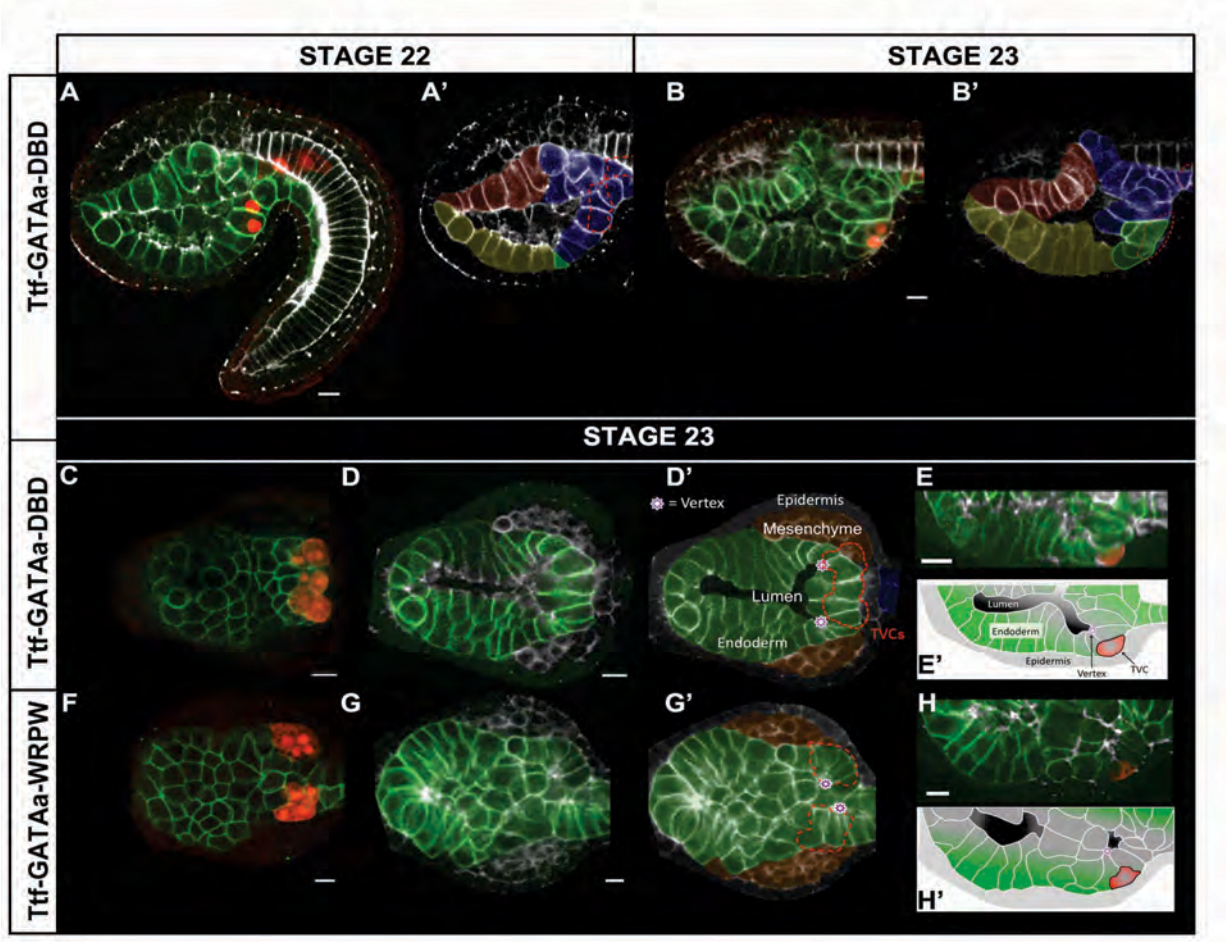


Figure 1. Association between heart precursors and endodermal cells. (A-H) Representative optical sections of embryos co-electroporated with Mesp-lacZ to mark the heart precursors (red), ttf-GFP-strabismus to mark the endoderm (green), and either ttf-GATAa-DBD (control) (A-E) or ttf-GATAa-WRPW (disrupts GATA in the endoderm) (F-H). Embryos were fixed at stages 22 and 23 as indicated and stained for F-actin (grey). (A-B) Lateral views of stage 22 and 23 embryos. (A'-B') Corresponding sections were pseudo-colored to illustrate the relationship between the heart precursors (red outline) and the hinge cells (green). (C-E) Ventral (C, D) and oblique (E) views of a representative embryo at stage 23. (C) Confocal plane showing the converged heart precursors and (D) the underlying endoderm. (D') Diagram of (D) illustrating the position of the heart precursors (red outline) in relation to the vertices (asterisk) of the hinge cells. (E, E') Oblique view of corresponding schematic illustration showing the association between the heart precursors and the hinge cells. (F-H') Representative ttf-GATAa-WRPW embryo imaged and schematized as described for (C-E'). Heart precursors fail to converge and underlying endoderm is abnormal when GATAa is disrupted. Scale bar: 12 um.

***Image created by Katerina Ragkousi, caption adapted from submitted paper: "A single GATA factor plays discrete, lineage specific roles in ascidian heart development," Katerina Ragkousi, Jeni Beh, Sarah Sweeney, and Brad Davidson.

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Professor of Biology: Bruce A. Hay
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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, microRNA function, 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 pharmaceutical or agricultural 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 and disease vectors. 3) Can we drive genes into wild populations so that all individuals express a trait of interest. With regard to this last aim, 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 1-3 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.

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

Ming Guo (and the Guo lab), Haixia Huang, Bruce A. Hay, Gal Barak, Kenneth Chan

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.

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64. 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.

65. Cell death, caspases and IAPs

H. Arno J. Müller, Bruce A. Hay, Chun-Hong Chen

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.

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

Haixia Huang, Shamili Allam, Joy Chen, Geoffrey Pittman

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? 5) Can we manipulate the biology of spermatogenesis so as to bias gamete production so that males produce gametes carrying the Y chromosome, but not the X chromosome? Elements with these characteristics, if they are located on the Y chromosome, are predicted to drive a population to extinction through the generation of male-only populations.

Reference

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

67. Cell death and the innate immune system

Chun-Hong Chen, Ming Guo, Bruce A. Hay

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.

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

Chun Hong Chen, Haixia Huang, Catherine Ward, Jessica Su, Nikolai Kandul, Geoff Pittman, Omar Akbari, Arun Kumar, Daniel Leighton, Bruce A. 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.

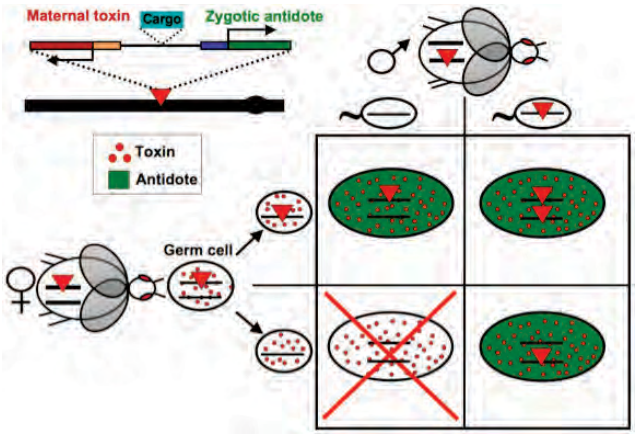

Figure 1. *Medea* is a "spiteful" selfish genetic element that enhances its transmission from generation to generation by causing the death of offspring that fail to inherit it. Mothers that carry a *Medea* element express a toxin (red dots) that is inherited by all oocytes (small ovals). Embryos (large 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. We imagine that *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. We are now expanding this work into the mosquito system.

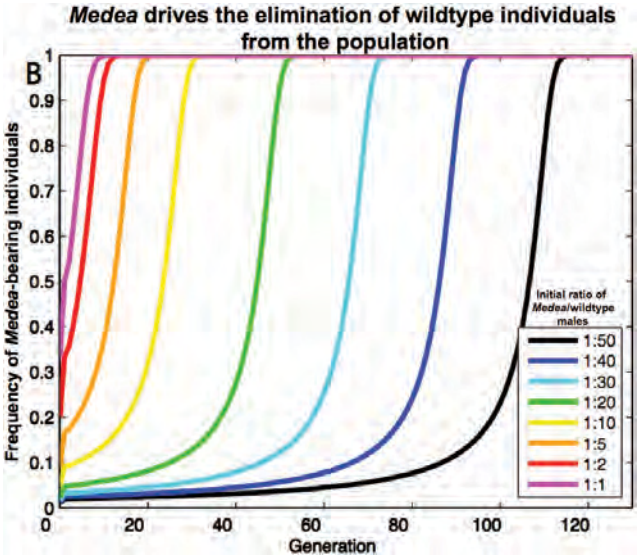

Figure 2. When *Medea*-bearing males are introduced into a population consisting of wildtype males and females, wildtype individuals are eliminated from the population. The greater the initial ratio of *Medea* to wildtype males, the more rapidly this elimination occurs.

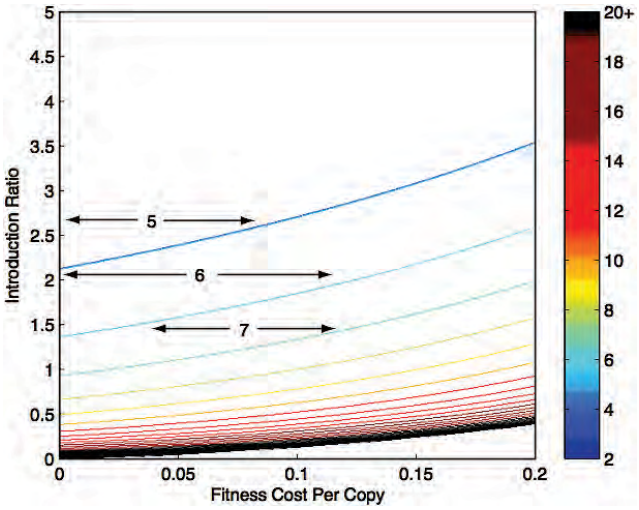

Figure 3. Medea's ability to spread, and the time it takes to become present in all individuals, is a function of fitness cost and introduction frequency. The plot describes the number of generations required for *Medea* to be present in 99% of individuals, for a *Medea* element with an embryonic fitness cost (resulting from the presence of a cargo transgene designed to protect from disease, for example). Homozygous *Medea*:non-*Medea* introduction ratios are indicated on the Y axis, and embryonic fitness cost on the X axis. Area between lines indicates regions of parameter space within which a specific number of generations (indicated by numbers and arrows) are required for the frequency of *Medea* individuals to reach a frequency of 99% or greater. Line color, shown in the heat map at right, provides a measure of how many generations are required. Black lines (50+) indicate that fifty or more generations are required. The border between the black-lined region and the lower unlined region defines the critical *Medea*:non-*Medea* introduction ratio, below which *Medea* will be eliminated from the population.

Reference

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

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

Kelly J. Dusinberre, Gal Barak

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.

70. Engineering reproductive isolation and population replacement using a synthetic underdominance system

Kelly Dusinberre, Katie Kennedy, Margaret Chiu, Jessica Su

The Medea system detailed above is very good at spreading genes into populations distributed over large areas, provided that modest levels of migration occur. This is ideal for situations in which the goal is to carry out population replacement in large regions. However, some communities may favor an approach in which population replacement is restricted to a local environment (Lets see how it does in your back yard, before trying it in mine). This creates a challenge: how to spread genes within a local environment, but maintain a barrier to migration-driven spread and fixation in surrounding regions. To address this need we are developing the synthetic underdominance system illustrated below. In this system homologous chromosomes carry toxin-antidote pairs in which the toxin present on chromosome A (Killer 1) is linked to an antidote (Rescue 2) that represses Killer 2. Killer 2 is located at the same position on the homologous chromosome B, linked with an antidote (Rescue 1) that represses Killer 1 (Figure 4). In such a system, organisms can only survive if they carry A and B chromosomes (in A/B individuals), or only wildtype (+) chromosomes (in +/+ individuals). A/+ and B/+ individuals die. A and B chromosomes will also carry genes that confer resistance to disease transmission. Such a system has two interesting features.

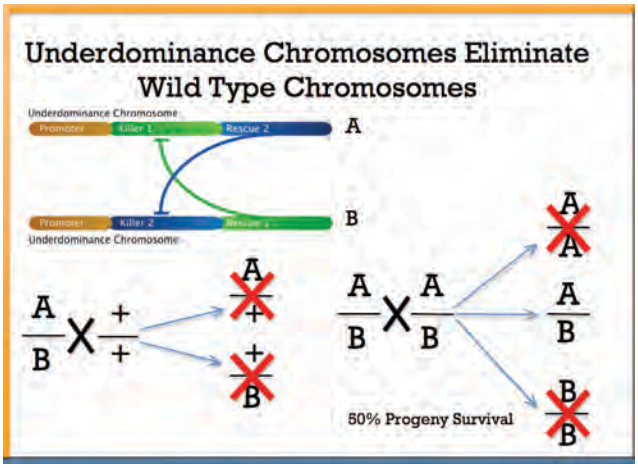

Figure 4. A single-locus underdominance system can be used to engineer reproductive isolation and population replacement.

First, it constitutes a simple method for engineering reproductive isolation (speciation). Matings between +/+ individuals produce viable progeny, as do matings between A/B individuals. However, mating between +/+ and A/B individuals produce only A/+ and B/+ progeny, which all die. This simple technology has a number of potential applications and provides a platform

from which to explore some of the evolutionary consequences of reproductive isolation.

Second, it provides a method for driving genes into a local environment in such a way that they are unlikely spread to fixation in surrounding regions through migration. In brief, for underdominance, as with Medea elements that carry a fitness cost, a threshold frequency must be achieved in order for spread to occur at all. With underdominance this threshold is quite high. But once the threshold is crossed, the underdominant system drives the wildtype chromosomes out of the population by causing their death in heterozygotes. A and B chromosomes also die in heterozygous progeny, but so long as A/B individuals make up greater than 66% of the population, more + chromosomes and thus, +/+ individuals are eliminated than are A and B chromosomes, in A/B individuals. The A/B genotypes have great difficulty in spreading into surrounding regions through migration because as these individuals migrate into areas composed largely of +/+ individuals, they are more likely to mate with +/+ individuals than with A/B individuals, resulting in the likely death of the A and B chromosomes in heterozygous progeny. We are developing several versions of underdominance in *Drosophila* and are working to move these systems to mosquito species.

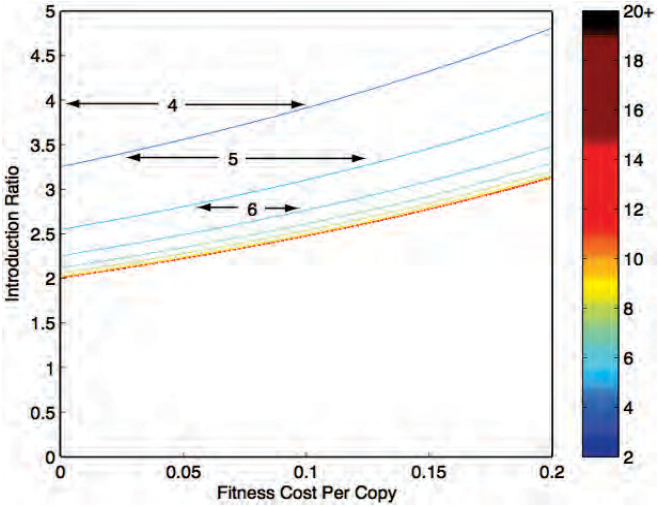

Figure 5. A single locus underdominant system's ability to spread, and the time it takes to become present in all individuals, is a function of fitness cost and introduction frequency. The plot describes the number of generations required for underdominant chromosomes to be present in 99% of individuals, for a situation in which the presence of the underdominant chromosomes is associated with an embryonic fitness cost (resulting from the presence of a cargo transgene designed to protect from disease, for example). Homozygous A/B:+/+ introduction ratios are indicated on the Y axis, and embryonic fitness cost on the X axis. Area between lines indicates regions of parameter space within which a specific number of generations (indicated by numbers and arrows) are required for the frequency of underdominant chromosome-bearing individuals to reach a frequency of 99% or greater. Line color, shown in the heat map at right, provides a measure of how many generations are required. Black lines (50+) indicate that fifty or more generations are required. The border between the red-lined region and the lower unlined region defines the critical A/B:+/+ introduction ratio, below which underdominant chromosomes will be eliminated from the population.

71. Sensing and responding to normal and abnormal microRNA expression

Nikolai Kandul, Alan Li

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression by suppressing the translation or promoting the degradation of transcripts to which they hybridize. Importantly for our purposes, when miRNAs are perfectly complementary to their target transcripts, transcript cleavage and degradation results. It is clear that miRNA expression is deregulated in many disease states. In addition, many viruses encode miRNAs that promote viral replication and/or suppress host defense systems. Our goal is to develop methods for sensing the expression of a particular miRNA, and then transducing this signal into changes in gene or protein expression. This will allow us to monitor the levels of miRNA expression in living animals. It will also allow us to regulate cellular physiology in response to the levels of particular miRNAs.

72. Predicting the fate of gene drive systems and their cargos in the wild

Catherine Ward, John Marshall

As we develop gene drive strategies we need to be able to predict how they are likely to behave. A number of question arise. Under what ecological and population genetic conditions will drive chromosomes spread? What are the likely epidemiological consequences of spread in terms of disease prevention? What are the likely functional lifetimes of these elements in the wild? What are the possibilities for removal and replacement of first-generation elements with second-generation elements? We are using mathematical modeling and computer simulations to address these issues for a number of different drive strategies.

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Research and Laboratory Staff: Taren M. Johnson, Jennifer Li, Sara W. McBride

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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.

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

Sung-Eun Lee, Vivian Yang

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.

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

June L. Round, Yun Kyung Lee, Arya Khosravi, Gloria Tran

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 microbiota, 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.

75. Affect of intestinal colonization with *Bacteroides fragilis* on autoimmune diabetes in NOD mice

Mary A. Yui, Sara W. McBride, Taren M. Johnson

Humans, and other organisms, are hosts to extremely complex, diverse and highly co-evolved microbial communities living at mucosal surfaces, especially in the gastrointestinal tract. Recent evidence has been accumulating which suggest that these microbial communities may play a key role in maintaining host health and avoiding autoreactivity, although mechanisms by which this occurs are as yet poorly understood. Correlations have been noted between the alarming rise in developed nations in the rates of autoimmune disorders, including Type 1 diabetes (T1D), and the decrease in human infectious diseases due to improvements in sanitation, vaccination, and the widespread use of antibiotics. While susceptibility to T1D has a strong genetic component in humans and rodent models, several lines of evidence in the rodent models suggest that manipulation of the microbiota, with pathogenic or non-pathogenic organisms, antibiotics, or diet, may reduce the incidence of T1D in animals genetically predisposed to develop diabetes. We are testing the hypothesis that a non-pathogenic human intestinal microbe, *Bacteroides fragilis*, can alter autoreactivity the non-obese diabetic (NOD) mouse model of T1D. *B. fragilis* is a prominent member of the human intestinal microbiota, and was the first symbiotic bacteria experimentally shown to affect the development of the mammalian immune system. *B. fragilis* has recently been shown by the Mazmanian lab to protect animals from experimental autoimmune colitis by inducing anti-inflammatory regulatory T cells through the production of a single product (Polysaccharide A or PSA). Induction of regulatory T cells has also been shown to be a powerful means of preventing T1D in NOD mice, so we are testing the hypothesis that mucosal colonization with this PSA-producing microbe can change the balance between pathogenic and protective T cells and prevent pancreatic inflammation and the development of diabetes in NOD mice. Groups of NOD mice have been colonized with *B. fragilis*, with and without PSA, using several different protocols and beginning at several different ages, to determine if there is any effect on the timing or incidence of diabetes. These studies are ongoing. In addition, we are testing if *B. fragilis* and/or PSA have direct effects on the types of cytokines secreted by dendritic cells and T cells isolated from NOD mice.

76. Immune modulation by microbial outer membrane vesicles (OMVs) during *Bacteroides fragilis* colonization

Yue Shen

Previous studies in the lab have shown *Bacteroides fragilis*, an important and numerically abundant commensal bacteria of the human gut, modulates host immune responses and prevents animals from experimental colitis. More importantly, the molecule that is required and sufficient for this immuno-modulatory activity has already been identified as one of the eight known capsular polysaccharides of *B. fragilis*, Polysaccharide A (PSA). In order to study the molecular mechanism for how PSA is delivered from *B. fragilis* to host immune cells, we proposed to investigate the potential role of outer membrane vesicles (OMVs), which are naturally produced by growing *B. fragilis*, in this delivery process. We have visualized OMVs produced by *in vitro* cultured *B. fragilis* via electron microscopy. And by immunoblot and immunogold labeling studies, and discovered that PSA is indeed associated with *in vitro* purified OMVs. Furthermore, our data show PSA-containing OMVs could induce specific immuno-regulatory response *in vitro* and could also protect animals from experimental colitis *in vivo* in a PSA-dependent manner, which suggested OMV-associated PSA has the same immuno-modulatory activity as purified PSA does. And the ongoing studies are aimed to further confirm these results, but more importantly, to test the necessity of OMVs in the delivery process as well as to investigate the detail processes of how OMV-associated PSA affects the physiology of the immune cells and eventually lead to the immuno-regulatory responses both *in vitro* and *in vivo*. A better understanding of the function of PSA associated with *B. fragilis* OMVs will not only help us to determine the role of OMVs in delivering PSA, but also shed light on the clinical application of *B. fragilis* OMVs in treating human inflammatory bowel disease.

77. Type VI secretion by *Helicobacter hepaticus* limits bacterial colonization and host intestinal inflammation during symbiosis

Janet Chow

Symbiotic bacteria colonize mammals by creating a molecular discourse with their hosts. Evolution has created numerous mechanisms that mediate these interactions, and bacterial secretion systems represent an important component for how microbes network with eukaryotic cells. Recently characterized, type VI secretion systems (T6SS) present a new means of forging microbial-host interactions. Interestingly, T6SS genes are present in over 25% of all bacterial genomes sequenced to date, yet specific knowledge of the function and substrates for these secretion systems remain largely unknown. Although mostly implicated in a role for virulence, a growing number of studies have shown type VI secretion may also mediate non-pathogenic relationships between microbe and host. Our study focuses on the role of the T6SS in *Helicobacter hepaticus*, a Gram-negative bacterium that stably colonizes the murine gastrointestinal tract for long-term without leading to intestinal disease. We find that mutants defective in T6S display higher intracellular and cell-associated numbers of bacteria upon incubation with intestinal epithelial cells *in vitro*. In addition, T6S mutants show increased colonization of the gastrointestinal tract *in vivo*. Furthermore, *in vivo* colonization with a T6SS mutant leads to exacerbated host inflammatory responses in an experimental murine model of colitis. Based on these findings, we propose that type VI secretion in *H. hepaticus* may function to maintain balance of a commensal relationship within the host gastrointestinal tract by limiting bacterial colonization and host inflammatory responses.

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Summary: Our laboratory has the goal of understanding the mechanisms of plant development, using both experimental and computational methods to test hypotheses. Land plants develop in two directions, up and down – with up being the shoot and its accompanying leaves and flowers, and down the root. We concentrate on the shoot, and on the set of stem cells that continuously provides the cells for the shoot throughout the growth of the plant. This set of cells is called the shoot apical meristem. It utilizes a number of different pattern-forming processes that are as yet poorly understood. The work reported below reviews some of our progress on a series of questions raised by the maintenance of the stem cell population in the shoot apical meristem of the plant model system *Arabidopsis thaliana*. One direction is to understand chemical signaling between the cells in the shoot apical meristem, as it is clear from mutant studies that at least three systems of chemical communication are essential for the maintenance and control of cell division and gene expression patterns in the shoot meristem. One of these systems is mediated by a peptide signal perceived by a transmembrane receptor kinase (called CLAVATA1, and therefore this system is termed the CLAVATA system). A second requires signaling by small molecules of the class called cytokinins, which are perceived by histidine kinase receptors similar to bacterial chemotaxis receptors. The third set of signals in the shoot meristem are communicated by the plant hormone auxin, another small molecule – but in this case the molecule not only diffuses passively, as does cytokinin, but also has an active transport system whose dynamic properties lead to pattern specification. Finally, we have also shown recently that the cells in the shoot meristem communicate by physical, as well as chemical signals – each cell can sense the principle direction of stress imposed by its neighbors, and this leads not only to cytoskeletal rearrangements that control cell wall synthesis, but also to changes in the position of the auxin transporter. Therefore there is feedback between the chemical and the physical signaling systems. We also study the control of cell division and gene expression in developing flowers, which are products of the shoot apical meristem, and which share some of the meristematic control mechanisms. Encapsulating the dynamic data and feedback between different modes of signaling in these developing tissues has led us to develop mathematical models of plant development, in which the dynamic data we gain from live imaging of growing plant tissues leads to hypotheses expressed as sets of equations, which when solved in a computer model the processes occurring in the real plant. The results from the computer are then used to predict experimental results, and new results are used to refine and alter the models. This iteration brings us closer to robust models of development, and therefore to an understanding of developmental principles. We call this approach to developmental biology Computational Morphodynamics.

78. Ligand-induced internalization of the CLAVATA1 transmembrane receptor kinase

Zachary Nimchuk

Cell numbers in above-ground meristem types of plants are thought to be maintained by a feedback loop driven by perception of the 13 amino acid glycopeptide CLAVATA3 (CLV3) by both the CLAVATA1 (CLV1) receptor kinase and the CLV2/CRN receptor-like complex. CLV3 made in the stem cells at the meristem apex limits the expression level of the stem cell-promoting homeodomain protein WUSCHEL (WUS) in the cells beneath, where CLV1 as well as WUS RNA is localized. WUS downregulation nonautonomously reduces stem cell proliferation. High-level overexpression of CLV3 eliminates the stem cells and causes meristem termination, and loss of CLV3 function allows meristem over proliferation. There are many open questions regarding the CLV3/CLV1 interaction, including where in the meristem it occurs, how it is regulated, and how it is that a large range of CLV3 concentrations between null and extreme overexpression gives no meristem size phenotype (buffering). We are using genetics and live imaging to examine the cell biology of CLV1 in *Arabidopsis* meristematic tissue. We have found that CLV1 is a plasma membrane protein that is destabilized by CLV3, such that CLV3 causes CLV1 to traffic to lytic vacuoles. We also find, using internalization of CLV1 expressed from a ubiquitously expressed promoter as an assay, which CLV3 can diffuse broadly in meristems. Ongoing studies are characterizing the protein partners of CLV1 in its trafficking, and assessing the requirement for a number of activities involved in endocytosis for the ligand-induced internalization of the CLV1 protein.

79. CORYNE appears to be a pseudokinase, not a kinase

Zachary Nimchuk, Paul Tarr

The apical stem cell niche in plants is restricted by a ligand- and receptor-activated feedback loop that regulates accumulation of the transcription factor WUSCHEL (WUS). Binding of the secreted peptide ligand CLAVATA3 (CLV3) to the CLAVATA1 (CLV1) LRR serine-threonine kinase is central to this process. CLAVATA2 (CLV2), a TM LRR protein lacking an internal kinase domain, is also important for perception of CLV3 and other CLV3-like ligands throughout the plant. In *Arabidopsis*, CORYNE (CRN), a putative TM serine threonine kinase with a short extracellular domain, has been shown to act with CLV2 in this process and may regulate CLV2 localization (Müller *et al*., 2008; Bleckmann *et al*., 2010). It has been proposed that CLV2 and CRN functionally assemble into a signaling complex that functions as a receptor kinase in parallel to CLV1 (Müller *et al*., 2008). Protein sequence analysis and comparison with serine-threonine protein kinases, however, has indicated to us that CRN is a pseudokinase, and not a kinase. We are currently testing bacterially produced CRN proteins and protein variants to determine if there is a kinase activity associated with this potential pseudokinase.

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80. Boundary specification and maintenance between the shoot apical meristem and organ primordia

Xiaolan Zhang

Boundaries in developing shoot apices serve the purpose of delimiting regions of gene activity and of separating distinct organs as they develop. M-O boundaries (meristem-organ) are formed to separate plant organ primordia from the shoot apical meristem (SAM), whereas O-O boundaries (organ-organ) develop between individual floral organs to create space between them. Loss of boundaries can result in abnormal organ fusion, failure of SAM initiation and defects in SAM maintenance. However, little is known of when and how the boundaries are specified and maintained during plant development.

Using live imaging and a fluorescent reporter of the boundary marker gene *LATERAL SUPPRESSOR* (pLAS:LAS-GFP), the dynamic process of boundary formation in *Arabidopsis* was studied. All boundaries initiate from the L1 cell layer and extend to 3-4 cell layers deep. Sepal boundaries start from the side away from the inflorescent meristem (IM), whereas boundaries between flower primordia and IM experience a dynamic change**:** first appearing from the two edges, spreading to almost the whole primordium, then limited to the boundary region until lost in the end. The time of disappearance of the LAS signal corresponds with the time of appearance of expression of the meristem marker transcripts from the *SHOOT MERISTEMLESS* (*STM*) and *WUSCHEL* (*WUS*) genes, implying an identity change from boundary identity to meristem identity.

In order to identify factors controlling boundary formation, I made DEX inducible lines of *STM* and *ASYMMETRIC LEAVES1* (*AS1*). Transient overexpression of *STM* leads to aberrant boundary specification, while *AS1* ectopic induction has no obvious effect. Similar experiments will be performed using inducible inactivation lines of *STM* (p35S::STM ds RNAi-GR) to validate results from the above experiments. The effect of plant hormones on boundary formation has also been explored using serial hormone treatments. Despite cytokinin, auxin and gibberellins affecting the position and the shape of sepal boundaries, cytokinin has the most severe boundary defects when applied at the same concentration. Future research directions will focus on the mechanism of how boundaries are maintained, specifically, the relationship between *LAS*, *STM* and cytokinin during plant development.

81. *HANABA TARANU* (*HAN*) and *HAN* family genes coordinately regulate organ separation, floral organ specification and phyllotaxy

Xiaolan Zhang

HAN is one of the 30 members of GATA type zinc-finger proteins in *Arabidopsis*, and the only one in the GATA family so far that has been identified to regulate flower development, meristem organization and cell division. Through time-course whole genome oligonucleotide microarrays using a *p35S::HAN-GR* line, we found that induction of *HAN* causes repression of *HAN* itself and three additional HAN family genes: *HANL2* (*HAN-LIKE2*, At4g36620), *GNC* (*GATA, nitrate-inducible, carbon-metabolism-involved*, At5g56860) and *GNL* (*GNC LIKE*, At4g26150). This repression was verified by real-time RT-PCR. HANL2, GNC and GNL belong to the same subfamily as HAN (subfamily II). Previously, *GNC* and *GNL* have been shown to redundantly promote chlorophyll biosynthesis, and are directly repressed by floral homeotic genes *APETALA3* and *PISTILLATA*. HANL2 is closely related to HAN, however, its biological function is unknown.

Although single mutants or any combination of double mutants of *gnc, gnl* and *hanl2* have no obvious flower developmental phenotypes, double and triple mutants of *han* with *gnc, gnl* and *hanl2* showed progressive effects on sepal fusion, petal number, silique length, and carpel and branching abnormalities. Plants with mutations in *han* and two of the three *HAN* family genes (all homozygous or two homozygous with one heterozygous) are completely infertile with almost no petals, highly fused sepals, abnormal carpels and branching defects, indicating that *HAN* and *HAN* family genes share similar functions during organ separation, floral organ specification, and plant branching. Further, we found that simultaneous decreased function of multiple

HAN family genes leads to dramatic fused branching and abnormal phyllotaxy during the reproductive stage, using ubiquitous induction of *amiHANL2-GNC-GNL*, suggesting new players for phyllotaxis regulation. Overexpression of a microRNA targeted to the HAN-regulating gene At1g28310, a Dof-type zinc finger domain-containing transcription factor, displayed similar branching and phyllotaxis defects, implying a gene regulatory cascade controlling floral organ development and phyllotaxis. Currently, four more experiments are in progress: 1) *in situ* hybridization to the RNA products of *HANL2*, *GNC*, *GNL* and At1g28310 to check their expression patterns; 2) crosses of *amiHANL2-GNC-GNL* with *han2* to see quadruple mutant-like phenotypes; 3) crosses of *35S-At1g28310* with *han2* and *amiHANL2-GNC-GNL* to identify potential phenotypic complementation; and 4) performance of ChIP-PCR to see whether HAN interacts directly with *HANL2*, *GNC* and *GNL* regulatory sequences.

82. Regulation of WUSCHEL stem cell niche in the shoot apical meristem

Yun Zhou

The shoot apical meristem (SAM) in *Arabidopsis thaliana* is being used as a model system to uncover the role of cell-cell communication in the control of plant stem cell populations. In *Arabidopsis*, the maintenance of the stem cell pool in the SAM is achieved through the balance between the loss of cells to lateral organs and the stem, and the proliferation of stem cells within the meristem. This dynamic in the SAM is determined through feedback regulation between CLAVATA (CLV) and WUSCHEL (WUS) proteins. Mutations in *CLV* genes result in the enlarged SAM and an over-proliferated stem cell pool, while the *wus* mutant loses the ability to maintain the SAM. CLV signal restricts the expression domain of *WUS*, a homeodomain transcription factor gene expressed only in a few cells within the rib meristem (RM), which is also referred as the stem-cell organizing center. In turn, WUS activates *CLV3* expression in the overlying central zone (CZ). The molecular mechanism that underlies the CLV–WUS feedback loop in regulation of SAM dynamics, especially the uncoupled but related phenomena of cell fate respecification and long-range control of cell division in the meristem peripheral zone, is being examined. Several regulators of *WUS* are identified and characterized; their roles in the regulation of *WUS* expression domain are being further investigated through live imaging of the proteins and distinct cell types in combination with transient perturbations of the target genes. Their functions in control of stem cell populations, and the involvement of different pathways balancing *WUS* repression and activation, will be examined through molecular and genetic analyses, leading to a coherent model of cell-cell communication in maintenance of the WUS stem cell niche in plant development.

83. Activator inhibitor model of plant patterning

Sean P. Gordon, Vijay S. Chickarmane

Higher plants maintain continuous development throughout their life by closely regulating the process of cell differentiation. Cell differentiation is partly controlled by hormonal cues, which interface with gene function. Based on characterization of hormone signaling and patterning of gene expression during *de novo* shoot meristem initiation from tissue culture, we propose a novel activator/inhibitor model by which auxin and cytokinin interact to regulate patterning of cell differentiation. In this model, the activity of auxin, the activator of cell differentiation, is regulated by cytokinin, an inhibitor of cell differentiation. Computational models of these interactions lead to self-organized patterning of hormone response and cell differentiation as observed in experiments.

84. A dynamic model for apical-basal patterning of the shoot stem cell niche during growth by cytokinin signaling

Vijay S. Chickarmane, Sean P. Gordon

A central unanswered question in stem cell biology, both in plants and in animals, is how the spatial organization of stem cell niches are maintained as cells move through them. We previously addressed this question for the shoot apical meristem (SAM) that harbors pluripotent stem cells responsible for growth of above ground tissues in flowering plants (Fletcher and Meyerowitz 2000; Sablowski 2007). We found that localized expression of receptor for the plant hormone cytokinin establishes a spatial domain in which cell fate is respecified through induction of the master regulator *WUSCHEL* as cells are displaced during growth. In our current work, we develop a computational model including feedback between apical stem cells, which produce active forms of cytokinin, and basal cells that perceive cytokinin. We show that these interactions are sufficient for relative positioning of cell fate in simulations of a dynamic growing model of the SAM. Within the SAM, cytokinin receptor expression extends basally relative to *WUS* expression. We propose that limited cytokinin availability limits *WUS* expansion in basal cells that express cytokinin receptor. Consistent with this hypothesis, perturbations with excess cytokinin result in *WUS* induction in basal regions of the cytokinin receptor domain. We further show experimentally and computationally that cytokinin promotes SAM growth.

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85. Control of the shoot stem cell niche through multiple antagonistic cytokinin receptors

Sean P. Gordon, Vijay S. Chickarmane

Cytokinins are plant hormones with diverse roles in growth and development, including promotion of SAM size and activity. The plant hormone cytokinin is perceived through three receptors in the model plant *Arabidopsis*. Although potentially redundant in some contexts, evidence indicates that individual receptors may specifically activate downstream genes (Kim, Ryu *et al*., 2006). We have recently shown that the cytokinin receptor AHK2/AHK4 is specifically required for cytokinin-mediated induction of stem cell associated phenotypes and WUS expression, a key gene positive regulator of SAM activity. Here, we perform a genome-wide study of gene expression in wild-type, *ahk2* and *ahk3* mutant plants using RNA-Seq. We show that AHK2 and AHK3 have a number of unique downstream targets, many of which are regulated in an antagonistic manner by AHK2 and AHK3. Furthermore, genes that are unique to AHK2 and AHK3 pathways are regulated in opposing manners by cytokinin treatment. Genes unique to AHK2 are more likely to be activated by cytokinin treatment than genes unique to AHK3, indicating that the AHK2 and AHK3 receptors may act through different mechanisms to regulate gene expression in response to cytokinin.

Publication

Gordon, S.P., Chickarmane, V.S., Ohno, C. and Meyerowitz, E.M. (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. *Proc. Natl. Acad. Sci. USA* **106**:16529-16534.

86. Patterning and maintenance of cell populations within the *Arabidopsis thaliana* shoot apical meristem stem cell niche by a hormone gradient

Paul Tarr, Elliot Meyerowitz

An essential part of plant and animal development is the establishment and maintenance of undifferentiated pluripotent stem cell populations within specialized proliferative tissues called stem cell niches. The stem niche represents a defined anatomical structure that serves as a reservoir of stem cells, which are responsible for the growth, homeostasis, and repair of tissues in an organism. Within the shoot apical meristem (SAM) of *Arabidopsis thaliana*, cell identity is not an inherent cellular property but a function of the positional cues a cell receives in the zone in which it resides (*1*). The stem cell niche of the shoot apical meristem is divided into three distinct zones of activity largely defined by their gene expression profiles and rates of cell division. The 20-30 cells in the center of the first two to three cell layers (L1-L3, primarily L1-L2) at the tip of the SAM constitute the Central Zone (CZ) and constitute the pool of pluripotent stem cells and are the ultimate source for all the cells in the above ground parts of the plant. In rice (*Oryza sativa)* the *LONELY GUY* (*LOG*) gene is expressed in the CZ and required for SAM maintenance. The *LOG* gene functions as a phosphoribohydrolyase by liberating the isoprenoid N^6 substituted adenine base (the active phytohormone) from the phosphopentose moiety of AMP in the terminal step of cytokinin biosynthesis (*2*). Cytokinins are implicated in a host biological processes including control of cell division and shoot initiation. The focus of this project will on characterizing the spatial expression patterns for the genes involved in cytokinin metabolism. Results from preliminary imaging studies suggest that there are discrete zones of cytokinin synthesis and degradation/inactivation within the SAM. We hypothesize that these zones of cytokinin synthesis and degradation within the SAM establish a gradient of cytokinin, which is required for the patterning, specification, and maintenance of the stem cell populations in the SAM of *Arabidopsis thaliana.*

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87. CYCLINs that regulate giant cell formation in *Arabidopsis* sepals

Christine Wu, Adrienne H.K. Roeder

While developmental patterning is found in all species, very little is known about how the cell cycle factors into the differentiation of specific cell types. *Arabidopsis thaliana* sepals have cells that range from one hundredth to one fifth the length of the sepal itself. These larger cells, called giant cells, form due to the cell undergoing endoreduplication as opposed to mitotic division (Roeder *et al*., 2010). We have previously shown that *LOSS OF GIANT CELLS FROM ORGANS (LGO)* controls giant cell formation in the sepals. LGO is closely related to *SIAMESE*, a gene that regulates endoreduplication in the trichomes of *Arabidopsis thaliana* by inhibiting D-type CYCLINs and CYCLIN-DEPENDENT KINASE (CDK) complexes (Churchman, *et al*., 2006). Thus, we began to search for CYCLINs that are downstream to LGO and interact with it. To do so, we examined CYCLIN overexpression plants to determine which CYCLINs were most likely to produce phenotypes resembling the *lgo* phenotype. These CYCLINs would be the best candidates for being inhibited by LGO. Among all the CYCLINs tested, overexpression of D-type CYCLINs produced sepals lacking giant cells. Therefore, we are examining double mutants between D-type CYCLINs and *lgo* to determine which combination will restore giant cell formation. Since D-type CYCLINs are likely to have redundancy in their functions, several cyclins might be controlling giant cell formation. To date, neither *cyclin D3* nor *cyclin D1* are sufficient to restore giant cell formation in *lgo* mutants. We continue our search for CYCLINs downstream of LGO to determine how giant cell patterning is regulated in the cell cycle.

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88. The epidermal specification pathway regulates giant cells in *Arabidopsis*

Adrienne H.K. Roeder, Carolyn K. Ohno

The division and growth of cells play an important role in pattern formation. How division and fate specification are coordinated can be addressed in the patterning of the outer surface of the *Arabidopsis* sepal, which contains a range of cell sizes from giant cells reaching a fifth the length of the sepal to cells as small as one-hundredth the length of the sepal. We first asked whether cells of different sizes have different fates as assayed by having different gene expression patterns. We have found one enhancer trap line that is expressed in giant cells and one expressed in small cells, suggesting that these cell types can be distinguished at the level of enhancers. We have previously shown that the variability in the timing of cell division creates this cell size pattern (Roeder *et al*., 2010). Now we ask whether such an apparently random mechanism is developmentally regulated. In a random mutagenesis screen for plants deficient in the production of giant cells, surprisingly we identified alleles of three members of the epidermal specification pathway: *atml1-2*, *dek1-4*, and *acr4-24*. *ATML1* encodes a homeodomain leucine transcription factor that is required together with its redundant partner *PDF2* for epidermal specification. *DEK1* encodes a transmembrane calpain protease and complete loss-of-function alleles are embryo lethal and lack an epidermis. *ACR4* encodes a transmembrane receptor kinase and mutations in the maize homologue *CR4* cause the epidermal cells to become enlarged and irregular. All of the alleles isolated in the screen have an intact epidermis and are deficient primarily in giant cells. We found that the expression of the giant cell marker is nearly absent in *dek1* mutants suggesting that *DEK1* controls giant cell identity. In contrast, the giant cell marker is still expressed in some small cells of *atml1* mutants, indicating that these mutants retain giant cell fate regardless of cell size. We conclude that the epidermal specification pathway has another role in controlling the cell size pattern.

Publication

Roeder, A.H.K., Chickarmane, V., Cunha, A., Obara, B., Manjunath, B.S. & Meyerowitz, E.M. (2010) Variability in the control of cell division underlies sepal epidermal patterning in *Arabidopsis thaliana*. *PLoS Biol.* **8**, e1000367. doi:1000310.1001371/journal.pbio.1000367.

89. Characterization of perivascular stem cells in multiple *Arabidopsis* organs

Kaoru Sugimoto, Yuling Jiao

Unlike most animal cells, plant cells can easily regenerate new tissues from a wide variety of organs when properly cultured. The common elements that provide varied plant cells with their remarkable regeneration ability are still largely unknown. Previously, we have characterized the initial process of *Arabidopsis in vitro* regeneration, where a pluripotent cell mass termed callus is induced (Sugimoto *et al*., 2010). From the results of our live imaging and mutant analysis, we found that callus from multiple organs resembles enlarged lateral root meristems and that similar to lateral roots, callus emerges from pericycle-like cells present around the vasculature of aerial organs. Previously, these pericycle cells had been described only in roots and hypocotyls of higher vascular plants. Also, the fact that a certain type of cells preferentially contribute to regeneration similar to animal tissue stem cells contradicts the previous belief that all plant cells are totipotent and regenerate new organs. Given these facts, we next aim to characterize these pericycle-like perivascular stem cells in multiple organs. To determine which tissues contain the pericycle-like cells and where these cells are located relative to the vascular components, we plan to follow a couple of pericycle fluorescent markers and determine the presence and position of pericycle-like cells in multiple organs. Using transmission electron microscopy (TEM), we will also characterize the cells morphologically. Thirdly, to answer the questions of what molecular mechanisms contribute to the competency of these cells to proliferate and differentiate upon callus induction, we will perform cell type-specific RNA sequencing. Generation of transgenic plants carrying the constructs for tissue-specific RNA isolation is now in progress.

90. Analysis of the process of shoot regeneration from callus in *Arabidopsis*

Kaoru Sugimoto, Sean Gordon

We previously characterized the callus formation process in the commonly used *in vitro Arabidopsis* regeneration system. In this system, first, a pluripotent cell mass (callus) is formed from a small piece of tissue 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. Surprisingly, our study revealed that callus resembles the root tip meristem regardless of the tissue of origin, which clearly shows that callus formation is not a simple reprogramming process backwards to an undifferentiated state as was widely believed. Therefore, callus tissue is not a homogeneous undifferentiated cell population, and the formation of shoot meristems from callus is the process of *trans*-differentiation from root-like to shoot. These conclusions raise questions regarding which cells give rise to shoot tissues, among the heterogeneous cell of root meristem-like tissue, and how they accomplish the root-to-shoot transition. To answer

the first question, now we are establishing techniques for cell lineage tracing, by marking cells conditionally using a heat-shock promoter. We also plan to observe the expression of root tissue markers and shoot primordium markers simultaneously, to find out whether shoot primordia arise from specific type of cells. For the latter question, of how a shoot is formed from root-like tissue, we are focusing on epigenetic-related genes that we have shown are up-regulated in callus compared to original tissues. In plants and animals, epigenetic regulators have been found to play roles in dynamic regulation of transcriptional outputs when the cell fate changes, by modifying chromatin structure. We have selected several mutations disrupting epigenetic-related genes, which cause abnormal shoot formation from callus. Further analysis will be done on these lines to reveal the role of chromatin remodeling in regeneration.

91. Cell-type specific analysis of translating RNAs in developing flowers reveals new levels of control

Yuling Jiao

Determining both the expression levels of mRNA and the regulation of its translation is important in understanding specialized cell functions. Here we describe both the expression profiles of cells within spatiotemporal domains of the *Arabidopsis thaliana* flower and the post-transcriptional regulation of these mRNAs, at nucleotide resolution. We express a tagged ribosomal protein under the promoters of three master regulators of flower development. By precipitating tagged polysomes, we isolated cell type-specific mRNAs that are likely translating, and quantified those mRNAs through deep sequencing. Cell type comparisons identified known cell-specific transcripts and uncovered many new ones, from which we inferred cell type-specific hormone responses, promoter motifs and coexpressed cognate binding factor candidates, and splicing isoforms. By comparing translating mRNAs with steady-state overall transcripts, we found evidence for widespread post-transcriptional regulation at both the intron splicing and translational stages. Sequence analyses identified structural features associated with each step. Finally, we identified a novel class of non-coding RNAs associated with polysomes. Findings from our profiling lead to new hypotheses in the understanding of flower development.

92. Dissection of the floral initiation pathway using genetic and genomic approaches

Wuxing Li

Understanding of the regulation of fate determination and patterning in organisms including plants and animals requires insight into their genetic regulatory networks. The shoot apical meristem of *Arabidopsis* provides an excellent system to study processes in cell division, cell differentiation, and cell fate determination. Furthermore, knowledge of the regulation of the flowering process is of agricultural importance, in addition to answering fundamental questions in biology. I am systematically characterizing the floral transition utilizing both genetic and genomic approaches. The transcription network 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 floral transition will be incorporated to build mathematical models to 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 genes whose expression is regulated at the chromatin level, will be determined.

93. Genetic studies of the expression pattern of *LEAFY*

Wuxing Li

Recent studies have identified the *Arabidopsis* gene *LEAFY* as a master regulator in specifying floral meristem identity. I propose a detailed characterization of this gene, including studies of : 1) factors that control the temporal and spatial pattern of *LEAFY* expression; 2) the upstream components of *LEAFY* regulatory function; and 3) genes that interact genetically or biochemically with LEAFY. The expression pattern of *LEAFY* has been observed in different mutant backgrounds and an auxin function-related gene was found to potentially regulate *LEAFY* spatial expression. Through mutagenesis studies in the background of the weak *lfy-5* allele, I have obtained several putative genetic modifiers, and further characterization of the interaction between *LEAFY* and these modifiers is providing new information toward understanding the floral initiation pathway. In addition, the function of chromatin regulatory components in the floral transition and in floral organ development is being investigated by genetic and genomic approaches. It is also expected that yeast one- and two-hybrid experiments will elucidate new components in the pathway of LEAFY function. This research will advance our understanding in fundamental questions such as cell fate determination and will impact on research in other multicellular organisms by answering fundamental questions about development.

94. A high-resolution spatiotemporal map for flower development by translatome profiling

Ying Wang, Yuling Jiao

In multi-cellular organisms, only subsets of genes are expressed during certain stages as well as in specific cell populations and they compose specific local gene regulatory networks (GRN). To reveal local GRN underlying flower development, we have adapted a methodology to immunoprecipitate polysomes as well as the associated mRNAs (Zanetti, *et al.*, 2005) in cell- and tissue-specific manners. By mRNA-sequencing analysis, we expect to reveal unique gene expression patterns in each cell group at different floral stages. Fourteen promoter lines are utilized for cell- and tissue-specific translation profiles, or translatome analysis. These promoter lines drive expression in the ABC floral domains (*pAP1*, *pAP3* and *pAG*), floral meristems (*pWUS*, *pCLV3*, *pSTM* and *pUFO*), abaxial/adaxial tissues (*pFIL* and

pZPR3), whorl boundaries (*pLAS* and *pPTL*), provascular tissues (*pAtHB-8* and *pAtHB-15*) and epidermal tissues (*pAtML1*). In order to achieve high temporal resolution, we rely on an AP1-GR *ap1 cauliflower (cal)* floral induction system to collect synchronized flowers (Wellmer *et al.*, 2006). We propose to analyze translatome profiles in specific cell domains in early floral stages 2-7 (flower stages according to Smyth *et al.*, 1990) using the floral induction system, and in late floral stages by hand dissection. From these experiments, we expect to reconstruct a high-resolution spatiotemporal translation map that may facilitate the modeling of local GRNs in terms of network topology, hub genes, and gene connections.

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Summary: The Rothenberg group studies the gene regulatory mechanisms that guide blood stem cells to ultimate fates as T lymphocytes. This developmental process is distinct from many of the developmental systems studied at Caltech, because hematopoietic stem cells provide a continuing source of new T-cell precursors throughout life, and development of new T-cell cohorts is mobilized in fetal life, neonatal life, and on through adulthood. This system is also distinctive because it is particularly good for shedding light on the stepwise choices the cells need to make in order to complete their differentiation as T cells. Blood precursor cells need to migrate to the thymus and expose themselves to sustained Notch1-Delta-like 4 (DL4) interactions in order to be triggered to differentiate into T cells. All the steps from multipotent precursor to committed T-lineage cell occur in this thymic environment, where they are relatively easy to isolate, characterize, and manipulate. Thus, we have been able to learn that these cells pass through a hierarchical decision tree that involves: the choice not to become a red blood cell or a platelet, the choice not to become a B cell, the choice not to become a macrophage or granulocyte, the choice not to become an antigen-presenting dendritic cell, and finally the choice not to become a natural killer cell, which leaves only various T-cell fates as the last options. This last decision concludes the T-lineage commitment process. The goal of research in this lab is to understand not only how the cells acquire the properties they will need to work as T cells, but also why the options that remain open to the precursors still are open, and how the cells make the decisions they do at each branch point. The answers we are interested in provide explanations in terms of specific transcription factor actions in gene regulatory networks.

A convergence of cell biological and molecular biological studies has revealed that the main events in early T-cell development can be broken into two major phases, split by the conclusion of commitment. Although both phases are normally dependent on Notch1-DL4 signaling, they involve different "jobs" for the cells. The first phase seems to encourage the precursors to proliferate, with limited acquisition of T-cell characteristics. The cells then cross the boundary into the second phase, when they seem to cut back on their proliferation and activate the full T-cell differentiation program. One of the regulators we have studied for many years, the Ets-family transcription factor PU.1, now emerges as a principal actor in the first phase. This factor can participate in gene regulatory networks pushing the cells to several different fates, but its early T-cell role is kept focused by interaction with Notch pathway signals. An exciting advance in the past couple of years has been identification of factors that control aspects of the transition from the first to the second major phase. One component of this transition is the repression of PU.1. We have gained insight into new cis-elements and unexpected deployments of trans-acting factors that probably cause PU.1 to be repressed during T-cell lineage commitment. We have also determined the identity of a factor that may be a major switch controller at the transition from phase 1 to phase 2, and this turns out to be the T-cell specific zinc finger factor Bcl11b.

Bcl11b is becoming a central interest for the group because of its distinctive regulation and function. This is perhaps the most T-lineage specific of all hematopoietic transcription factors, and the single one which is likely to be rate-limiting for commitment *in vivo* based on its expression pattern. We have shown that if Bcl11b is deleted, phase 1 pro-T cells fail to undergo commitment, spawn non-T cells abnormally even in the presence of Notch signals, and most intriguingly gain the ability to keep proliferating without developmental progression. The cis- and trans-elements required to turn Bcl11b on can be equated with those that define T-lineage identity, and so they are a major focus of our current work. Further, the mechanism through which Bcl11b works to bring about commitment involves identifying its own direct target genes and interaction partners, and that has become another important focus. Bcl11b's action at the last major identity determination point for T-cell precursors may involve network interactions with competing phase 1 regulators, and the gene regulatory network aspects of its role are another important project. Finally, by combining fluorescent reporters for PU.1

expression and Bcl11b expression, we have initiated a collaboration with the Elowitz group to study the lineage commitment process in individual T-cell precursors.

We began creating a provisional gene regulatory network model to account for the T-cell development pathway two years ago, based on the effects of transcription factor perturbation on the expression of multiple developmentally regulated genes. This process illuminated the need for three additional kinds of information in order to complete and confirm the model. First, we needed a more complete "parts list" for the T-cell specification process: we needed to know all the transcription factors and potential signaling systems that might be candidates for regulatory roles. Second, we needed a way to locate the candidate cis-regulatory sites at which these factors might work on their target genes. Third, we needed better tools for dissecting the roles of these factors via stage-specific loss or antagonism of function. All three of these needs are now being addressed through advances in the past year. Starting with the third need, we have begun to obtain highly revealing results using a systematic dominant-negative strategy that distinguishes direct from indirect transcription factor actions on affected target genes. To address the first and second needs, we have mounted a major survey of all the changes in both RNA expression and epigenetic histone marks throughout the genome as the cells progress from the earliest T-cell development stages to commitment and beyond. This enormous enterprise has been made possible by a collaboration with the Wold lab, and it has yielded a broad picture of the cis- and trans-regulatory changes that occur at each stage of the T-lineage specification pathway. With this rich resource, an abundance of tools is suddenly in hand to solve the nature of the T-cell commitment process.

The commitment process is not only a way for T-cell precursors to renounce other hematopoietic fates; it is also closely intertwined with poorly understood events that will go on to influence the subspecialization of T-cell fate that the cells will undertake, and even to determine whether or not they will be allowed to survive in the T-cell lineage. In the past six years we have identified variations in these early events in the T-cell precursors of autoimmunity-prone mice. Genome-wide transcriptome analysis now suggests that the autoimmunity-prone genetic background causes defects in phase 1 to phase 2 progression. Ultimately, the early defects may cast a shadow over the cells' future roles as mature T cells and contribute to autoimmunity.

95. Genome-wide correlation of histone modifications and gene expression during early T-cell development

Jingli Zhang

Through the collective activities of lineage and/or stage-specific regulators, external signaling is converted into epigenetic information that, in addition to helping maintain cellular identity, may influence cell fate decisions during development. Accumulated studies have demonstrated that temporal and spatial variations of histone modifications are closely associated with developmentally regulated gene expression. T-cell lineage commitment is marked by the gradual attenuation of stem cell-like self-renewal characteristic and alternative fate potentials, and the step-wise establishment of T-cell identity. To decipher the molecular mechanisms that underlie the gene regulation and the lineage decision during early T-cell differentiation, we investigated the genome-wide histone modification status and gene expression at different T cell development stages. We generated global maps of the active histone marks H3K9/14ac, H3K4me2, and the repressive mark H3K27me3, and mRNA for fetal liver derived DN1, DN2a and DN2b, and adult thymic DN3 and DP by deep sequencing (ChIP-seq and RNA-seq).

Our data showed that indeed as DN1 cells became more mature, a group of hematopoietic stem cell factors and alternative lineage regulators, including Lmo2, Lyl1 and Cebpα, were actively downregulated by the combination of gradually enhanced H3K27me3 and diminishing of H3K9/14ac on the promoter regions. As a result, we observed a pattern of gradual reduction in mRNA expression of those genes. A few downregulated alternative lineage genes, such as PU.1, did not show notable argument of H3K27me3 on the promoters, and we found those genes were instead silenced by the reduction of H3K9/14ac and H3K4me2. Reciprocally, a group of T-cell identity regulators, such as Bcl11b and the T-cell specific differentiation gene CD3ε, were steadily upregulated during the commitment process, paralleled with the elevation of H3K9/14ac and H3K4me2 on the promoters. Among those genes, some were initially repressed by H3K27me3. As expected, we observed a corresponding removal of H3K27me3 from these promoters. Interestingly, for most upregulated genes, the H3K4me2 mark was increasingly "loaded" on the TSS regions before the gene transcription was initiated, implying that H3K4me2 is a mark for developmentally labile genes.

Taken together, our data suggest that multiple regulation mechanisms are involved in controlling T-lineage commitment.

96. Role of Bcl11b in T-cell lineage commitment

Long Li

The most important process of T-cell lineage commitment is turning off the stem cell regulatory program and alterative lineage potentials by T cell precursors. It has been shown that the DN2 stage is where precursors complete the commitment step. Further analysis of DN2 by M. Yui has divided DN2 cells into two distinct populations: DN2a and DN2b. DN2a cells, which express a high level of Kit on their surface, are un-committed T cell precursors with alternative lineage potentials, whereas DN2b cells, which have lower levels of Kit, are committed T-lineage cells. Efforts have been spent to identify regulators in T cell lineage commitment between DN2a and DN2b. The transcription factor Bcl11b with its unique expression pattern turns out to be a candidate. C.C. Tydell and others in our lab have found that in hematopoietic tissue, the expression of Bcl11b is restricted to the T-lineage, and its mRNA level increases dramatically when cells developed from DN2a to DN2b, around the time that precursors commit their fate to T-lineage. Biochemistry studies have found Bcl11b is apparently a repressor because it interacts directly with histone deacetylases. Before we started this project, such evidence supported the hypothesis that Bcl11b could be a major regulator in T-lineage commitment by inhibiting developmental plasticity of T cell precursors. The objective of this project has been to address the role of Bcl11b in early T cell development.

The data we generated indeed proved our hypothesis. Using retroviral Cre and a conditional Bcl11b knockout mouse model, we showed that absence of Bcl11b blocked T cell development at a DN2a-like stage. These cells express high levels of transcription factors associated with stem-ness, such as SCL, Lyl1, Lmo2, Erg, Gfi1b and GM-CSFRb, and retain high levels of genes that involved in other lineages, such as *Bcl11a*, *Id2*, *Il2Rb*, *E4BP4* (*Nfil3*) and *Eomes*. When cultured on OP9-DL1 or OP9-control with certain cytokines, Bcl11b-deficient DN2a cells efficiently developed to NK-like cells and $Mac1^{+}Gr1^{+}$ myeloid cells, which is consistent with their gene expression pattern. Strikingly, although Bcl11b-deficient DN2a cells are unable to commit to T lineage, they express fair amounts of T cell identity genes including *Ptcra*, *Cd3e*, *Gata3* and *Tcf7*, an indication that T cell lineage specification initiates normally when Bcl11b is absent.

From our data, IL7Ra is apparently a target of Bcl11b in early T cells. Control DN2a cells develop to DN2b/3 cells in response to decrease of IL7 in culture media, whereas Bcl11b-deficient DN2a cells are IL7 independent. This culture system offers a good experimental design to study interactions of Bcl11b with the IL7 receptor-signaling pathway. Considering that both factors are major regulators of commitment, the results would increase our knowledge of T cell lineage commitment.

97. Cis-regulation of Bcl11b in T-lineage commitment

Long Li, Jingli Zhang, Hao Yuan Kueh

Based on our published results, it is known that Bcl11b is a major regulator of T cell lineage commitment. The next question is "who regulates the regulator." The answer would also help to address a more general issue in the field: the regulatory machinery that controls T cell lineage commitment. The timing and expression pattern of Bcl11b could be used here as a tool to study genetic inputs that regulate T cell lineage commitment.

First, we have located three CpG islands in Bcl11b locus, one of which is mapped to Bcl11b promoter region. The DNA methylation of promoter region was measured using bisulfite-DNA sequencing. The results indicate there is an unmethylated window from -900 to -00 bp of *Bcl11b* locus in Bcl11b expressing P2C2 cells (DN3 like pre-T cells) and Rag1 knockout thymocytes. As a control, the CpG island on Exon 4 is methylated in both Bcl11b-expressing cells and non-T cells. We then made several promoter-luciferase reporter constructs, but stable transfections of P2C2 cells and Raw264.7 cells (pre-myeloid cells) were unable to recapitulate Bcl11b expression.

A genome-wide mapping of chromatin modifications in pro-T cells showed that a Bcl11b downstream region (MP) shares the same chromatin modifications as the *Bcl11b* promoter. In a subset of human T cell leukemia, this region is involved in activating genes that translocate to *Bcl11b* locus due to chromosome breaks. Based on this evidence, our hypothesis is that the downstream region is a regulator of Bcl11b expression. To test the hypothesis, we first used chromatin conformation capture (3C) to map physical interaction between Bcl11b promoter and the downstream region. The results revealed a T-cell specific interaction between the region and Bcl11b promoter/exon1, suggesting that our hypothesis might be true. We then created a mini-gene by knocking MP into a Bcl11b-IRES-YFP BAC. The BAC constructs were transfected into T-lineage P2C2, myeloid-lineage Raw264.7 and nonhematopoietic NIH/3T3 cells. Initial results suggest that the inclusion of MP in this construct may play a role in restricting its activity to T-lineage cells, as the Bcl11b-MP-YFP BAC almost recapitulates the normal Bcl11b expression pattern in this system. If this is confirmed, it will locate a major *cis*-regulatory element for Bcl11b cell type specificity.

98. Investigating lineage commitment mechanisms in T-cell precursors using live-cell imaging

Hao Yuan Kueh

Haematopoietic progenitors maintain plasticity to alternative developmental fates during early T-cell development, but eventually decide to exclude alternate fates and commit to a T-lineage fate. Circuits of interacting genes and proteins are believed to enable progenitor cells to integrate developmental signals and decide between alternate fates; however, the mechanisms

by which these circuits mediate fate decision in making in progenitor cells remain unclear. In this project, we aim to gain insight into lineage choice mechanism by monitoring the expression of key regulatory genes involved in fate decision making using live-cell imaging. We focus on Bcl11b and PU.1, two transcription factors that play important roles in T-cell fate commitment. Bcl11b is sharply up-regulated during early T-cell development and plays a key role in promoting commitment (Li *et al*., 2010), whereas PU.1 is down-regulated during development and diverts T-cell precursors into a myeloid fate when present.

To facilitate live-cell measurements of Bcl11b expression, we have used conventional gene targeting to develop two transgenic mouse strains: 1) Bcl11b^{YFP}, which contains a Histone-2B (H2B)-mCitrine YFP gene inserted into the 3' UTR of the Bcl11b gene. This transgene reports on Bcl11b expression without affecting its endogenous levels; 2) Bcl11b^{RFP}, which contains a H2B-mCherry RFP gene inserted in place of exon1 of endogenous Bcl11b gene. This strain acts as a germline knockout that also reports on endogenous gene transcription. We have successfully generated the Bcl11b^{YFP} transgene mouse strain in collaboration with the Caltech transgenic mouse facility. Using flow cytometry, we found that Bcl11b is absent in early thymic progenitors (ETPs), begins to be expressed in a fraction of specified but uncommitted DN2a T-cell precursors (Yui *et al*., 2010), and remains expressed in all subsequent developmental stages, consistent with our previous studies (Tydell *et al*., 2007). These results validate the use of the Bcl11b^{YFP} reporter allele for live-cell imaging studies. For the Bcl11b^{RFP} allele, we have generated clones of correctly-targeted embryonic stem (ES) cells, and are currently injecting these cells into tetraploid embryos to generate transgenic mice.

For live-cell measurements of PU.1 expression, we are using transgenic PU.1-GFP reporter mice developed by Stephen Nutt and colleagues. In preliminary imaging experiments of PU.1-GFP progenitors cultured on O9-DL1 cells, we found that the activity of the PU.1 promoter decreases progressively in individual progenitors during T-cell development, consistent with results from flow cytometry (Nutt *et al*., 2005). We are now performing a detailed analysis of the imaging data to further characterize the kinetics of PU.1 down-regulation and understand its implications for fate decision making in single cells. In future studies, we will further probe mechanisms underlying dynamic PU.1 regulation by perturbing either signaling inputs or the expression of regulatory genes, and measuring resultant effects on PU.1 promoter activity in single cells. We will also examine regulatory relationships between PU.1 and Bcl11b by simultaneously measuring PU.1-GFP and Bcl11b^{RFP} expression in progenitor cells containing both reporter alleles. Through these experiments, we hope to gain insights into the behavior and functioning of the regulatory gene circuitry underlying lineage choice in haematopoietic progenitor cells.

99. Modification of PU.1 activity by Notch signaling and Bcl11b in early T-cell development

Marissa Morales Del Real

PU.1 is an Ets family transcription factor that is expressed in several types of hematopoietic cells including HSC, progenitor cells, and mature cells. Its regulated expression is especially important for the development of B cells, myeloid cells, and early T-cell development. The effects of PU.1 have been shown to be linked to its level of expression as well as to its binding/activity partners. During early T-cell development, PU.1 is expressed at high levels. We believe that by interacting with Notch signaling (or Notch target genes) and other transcription factors, PU.1 activity is modulated in such a way that these multipotent progenitor cells are driven to a T pathway. A cell line model has been used to identify candidate genes that interact with PU.1 to either drive cells to a myeloid pathway or to a T-cell pathway. QPCR analysis of sorted populations identified several genes important for T cell development that are down regulated with PU.1 over-expression; including *Gfi1*, *Bcl11b*, *Myb*, *HEBalt*, and *Gata3*. Co-infection experiments with PU.1 and these candidate genes suggest that HEBalt and myb may not be involved in the lineage decisions mediated by PU.1. However, the transcription factor, Bcl11b blocks the diversion of PU.1 over expressing cells. This suggests that Bcl11b may be involved in the modification of PU.1 activity during early T cell development. Subsequent QPCR analysis of sorted populations simultaneously over-expressing PU.1 and Bcl11b show that Bcl11b protects a subset of genes down regulated by PU.1 over expression and also prevents the up regulation of other genes normally up regulated by PU.1. We are now in the process of testing if any of these genes are involved in the PU.1 mediated diversion pathway or, alternatively, if they are involved in protection of PU.1 mediated diversion. We are also interested in investigating physical interactions of PU.1 and Bcl11b as well as exploring the PU.1/Bcl11b relationship in primary T-cell precursors.

100. Role of the transcription factor PU.1 in early T-cell development

Ameya S. Champhekar, Ellen V. Rothenberg

Thymus-seeding precursor cells progress through four CD4- CD8- double negative (DN) stages - DN1- DN4 - and in the process commit to the T-cell lineage between the DN2a and DN2b stages. Stage-specific expression and downregulation of several transcription factors is important for this process. The Ets family transcription factor PU.1 is expressed in the pre-thymic as well as the early DN1 and DN2a and DN2b stages. PU.1 expression is coincident with developmental plasticity of these stages and its expression is shut off when cells commit to the T-lineage. To study the role of PU.1 in the DN1 through DN2b stages, we used the following methods to perturb PU.1 function in e14.5 fetal liver precursors and FL derived DN cells: *i*) Cre recombinase expressing PU.1$^{fl/fl}$ cells to study loss of function; *ii*) over-express a construct

that has the DNA binding domain of PU.1 fused with the engrailed repressor domain (PU.1 engrailed), and *iii*) a PU.1 deletion construct lacking the transactivation domain (PU.1 ΔDEQ). Using these approaches we have shown that PU.1 expression is essential for survival and proliferation of c-Kit+ CD27+ FL precursor cells. This effect could be at least in part because PU.1 positively regulates expression of the cytokine receptor Flt3, which provides proliferation and survival signals to these cells. We have also found that PU.1 is important for proper DN progression as in the absence of PU.1 fewer FL precursor cells progressed to the DN2 stage as compared to WT cells.

Recently, we have obtained gene expression data from DN2a and DN2b cells over-expressing PU.1 WT and the PU.1-engrailed fusion construct in an effort to identify PU.1 target genes and thus its function in these stages. These data indicate that PU.1 may play an important role in the extended period of T-lineage plasticity between DN1-DN2a stages, apparently by positively regulating a transcriptional repressor(s) that suppresses T-cell lineage genes like *Tcf7*, *Gata3*, *Lef1*, *Rag1*, *HEBalt*, and *Spib* that are expressed at peak levels at the DN3 or later stages of T-cell development. At the same time, PU1 may also keep DN thymocytes on the path to becoming αβ T-cells by suppressing genes important for the development of γδ T (*Runx3*, *Plzf* (*Zbtb16*) and *Id3*), NK (*IL2Rb*) and Mast cell (*Gata2*, *Kit*) lineages. We are currently analyzing gene expression patterns from PU.1 ΔDEQ expressing and PU.1$^{-/-}$ DN1, DN2a and DN2b cells to help us identify important target genes and to complement our PU.1 engrailed data. Microarray analysis of PU.1-/- DN subsets will help us identify the repressor protein(s) regulated by PU.1 in these cells.

101. Cell-type-specific activation and repression of PU.1 by a complex of discrete, functionally specialized *cis*-regulatory elements

Mark A. Zarnegar, Ellen V. Rothenberg

The transcription factor PU.1 is critical for multiple hematopoietic lineages, but different leukocyte types require strictly distinct patterns of PU.1 regulation. PU.1 is required early for T-cell lineage development but then must be repressed by a stage-specific mechanism correlated with commitment. Other lineages require steady, low expression or upregulation. Until now, only the promoter plus a distal upstream regulatory element (URE) could be invoked to explain nearly all *Sfpi1* (PU.1) activation and repression, including bifunctional effects of Runx1. However, the URE is dispensable for most *Sfpi1* downregulation in early T cells, and we found that it retains enhancer activity in immature T-lineage cells even where endogenous *Sfpi1* is repressed. We have obtained evidence for another complex of conserved noncoding elements that mediate discrete, cell-type-specific regulatory features of *Sfpi1*, including a myeloid-specific activating element and a separate, pro-T-cell-specific silencer element. These elements yield opposite, cell-type-specific responses to Runx1. T-cell-specific repression requires Runx1 acting through multiple nonconsensus sites in the silencer core. These newly characterized sites recruit Runx1 binding in early T cells *in vivo* and define a functionally specific scaffold for dose-dependent, Runx-mediated repression.

102. *In vitro* systems for generation of early T-cell precursors from adult murine bone marrow

Amy A. Ross, Patricia Vegh, Rochelle A. Diamond, Margaret Ho, Diana Perez, Ellen V. Rothenberg

Our research has focused on developing *in vitro* culture systems for early T-cell lineage precursors that can be used in our gene network models. The ability to generate T-lineage cells from non-fetal tissues (e.g., thymus or liver) has the advantage of eliminating the logistical issues associated with timed mating for obtaining progenitor cell populations. Further, the availability of T-cell precursors that are derived from adult tissues may be useful in on-going studies that manipulate gene expression in T-cell differentiation pathways.

Our previous studies focused on *in vitro* systems for the generation of early T-cell precursors (DN1 - DN3) using thymocytes isolated from pre-weaned or young adult (6-8 weeks) wild type (C57BL/6), Rag knock-out (B6-Rag2$^{-/-}$), and Toll-like receptor deficient (TLR3$^{-/-}$) mice. Our *in vitro* system relies upon the stromal feeder cell line OP9-DL1 to provide the Notch signaling required for T-cell differentiation. Our early studies using this system showed that it is possible to generate DN1 through DN3 subsets from ETP and DN1-sorted cells from all three populations of mice. However, in order for the system to be useful in our gene network studies, the cultured thymocytes must be able to withstand gene transfection procedures.

Our laboratory has reported extensively on the successful use of retroviral vectors for gene manipulation in fetal-derived thymocytes. However, attempts to use nucleofection protocols (Amaxa Nucleofection®) with morpholinos and siRNA on DN1-DN3 cells derived from non-fetal derived T-cell progenitors have met with mixed results. Our earlier studies on thymic DN1-DN3 cells derived from pre-weaned or young adult mice described above concluded that although transfection is possible using the Amaxa Nucleofection® protocol, the survival rate of transfected cells is too low to be useful. This appeared to be true regardless of the vector used, whether it was morpholinos, siRNA, or GFP-negative controls.

In an attempt to generate adequate numbers of early T-cell lineage precursors *in vitro*, we adapted our culture system for cells isolated from adult (12-16 weeks) C57Bl6 bone marrow. Our initial attempts used lineage-depleted/ Sca-1 high/c-kit high-sorted marrow cells plated onto the OP9-DL1 cultures. After six days in culture, cells were transferred onto fresh OP-9 DL1 cells to maintain Notch signaling. After a total of 10 days in culture, analyses by flow cytometry revealed bountiful populations of DN2 –DN3 cells. Additional experiments showed that cell sorting for progenitor cells was not necessary for the

in vitro proliferation of DN2 – DN3 cells. Hence, the system was modified to use only lineage-depleted, unsorted cells and results were comparable to previous studies.

Early data on the use of the Amaxa Nucleofection® protocol in presumptive DN2 cells derived from adult marrow are encouraging. In contrast to the cell death observed in our studies on thymus-derived T-cell precursors, the marrow-derived precursors appear to tolerate the procedure with superior rates of transfection with various siRNA's. To date, we have tested the system with GATA-3, c-myb, Pax-5, and Risc-free and GFP empty-vetor control siRNA nucleofection. Nucleofection rates for all groups are similar, with transfection percentages averaging well above 30%. Further, the nucleofected cells are capable of sustained *in vitro* viability on OP9-DL1 cultures up to 48 hours post-treatment. However, flow cytometric analyses for effects of gene-knockdown of known transcription factors (e.g., GATA-3 siRNA) have not shown any discernible phenotypic changes amongst the groups. Current studies are focused on gene typing analyses of sorted nucleofected cells within the DN2- DN3 phenotype versus non-nucleofected and thymus-derived T-cell precursor controls. These studies will allow us to determine if the *in vitro* system does indeed generate true T-cell precursors and if gene knockdown is achievable in this system.

In conclusion, the availability of an *in vitro* assay for generation of early T-cell precursors from adult marrow has the potential to overcome some of the obstacles and costs associated with obtaining cells from fetal tissue sources. Further, if the assay also provides for a more robust population of cells that can withstand nucleofection procedures, this system has the potential to provide an additional research tool for our gene network models in T cell development.

103. Fine-scale staging of T-cell lineage commitment in adult mouse thymus

Mary Yui, Ni Feng

T cell development is marked by the loss of alternative lineage choices accompanying specification and commitment to the T cell lineage. Commitment occurs between the CD4 and CD8 double-negative (DN) 2 and DN3 stages in mouse early T cells. To determine the gene regulatory changes that accompany commitment, we sought to distinguish and characterize the earliest committed wild type DN adult thymocytes. A transitional cell population, defined by the first down-regulation of surface c-Kit expression, was found to have lost the ability to differentiate into dendritic cells (DC) and natural killer (NK) cells when cultured without Notch-Delta signals. In the presence of Notch signaling, this subset generates T lineage descendants in an ordered precursor-product relationship between DN2, with the highest levels of surface c-Kit, and c-Kit-low DN3 cells. These earliest committed cells show only a few differences in regulatory gene expression as compared to uncommitted DN2 cells. They have not yet established the full expression of Notch-related and T cell differentiation genes characteristic of DN3 cells before β-selection. Instead, the downregulation of select stem cell and non-T lineage genes appears to be key to the extinction of alternative lineage choices.

104. Divergence of αβ and γδ lineages at the first TCR dependent checkpoint: preferential γδ and impaired αβ T-cell development in non-obese diabetic (NOD) mice

Ni Feng, Patricia Vegh, Mary Yui

The first TCR-dependent checkpoint in the thymus determines αβ vs. γδ T lineage fate and sets the stage for later T cell differentiation decisions. We had previously shown that the early T cells in NOD mice that are unable to rearrange a TCR exhibit a defect in checkpoint enforcement at this stage. To determine if T cell progenitors from wild type NOD mice also exhibit cell-autonomous defects in development we investigated their differentiation in the Notch-ligand presenting OP9-DL1 co-culture system, as well as by analysis of T cell development *in vivo*. Cultured CD4 and CD8 double negative (DN) cells from NOD mice exhibited major defects in the generation of CD4 and CD8 double positive (DP) αβ T cells, while γδ T cell development from bipotent precursors was enhanced. Limiting dilution and single cell experiments show that the divergent effects on αβ and γδ T cell development did not spring from biased lineage choice but from increased proliferation of γδ cells and impaired accumulation of αβ lineage DP cells. *In vivo*, NOD early T cell subsets in the thymus also show characteristics indicative of defective β-selection, and peripheral αβ T cells are poorly established in mixed bone marrow chimeras, contrasting with strong γδT as well as B cell repopulation. Thus, NOD T cell precursors reveal divergent, lineage-specific differentiation abnormalities *in vitro* and *in vivo* from the first TCR-dependent developmental choice point, which may have consequences for subsequent lineage decisions and effector functions.

105. Genome-wide genetic analysis of an early T-cell checkpoint violation in NOD mice

Mary Yui, Ni Feng, Jingli Zhang

Non-obese diabetic (NOD) mice spontaneously develop Type 1 diabetes (T1D). Over 20 diabetes susceptibility loci have been found to contribute to autoimmunity in this model of diabetes. T cells play a critical role in T1D pathogenesis in both mice and humans, and several developmental abnormalities have been reported in the early T cells from NOD mice, including an early β-selection checkpoint defect. Progenitor cells from NOD mice with mutations that prevent T cell receptor (TCR) rearrangements do not arrest at this TCR-dependent checkpoint, unlike early T cells from other mouse strains. The phenotype of these breakthrough cells shows characteristics of the spontaneous transduction of a partial TCR signal. A genome-wide quantitative trait locus (QTL) linkage analysis was conducting using an F2 cross

and N2 backcross and SNP genotyping to determine if this checkpoint violation maps to known T1D susceptibility loci in NOD mice. Remarkably, the three most prominent QTL regions found in these crosses map to previously identified diabetes susceptibility regions, including those on chromosome (chr) 4 (with diabetes susceptibility loci *Idd9* and *Idd11),* chr 17 (*Idd1), and* chr 11 (*Idd4*). In parallel with the QTL mapping, we have carried out a genome-wide RNA deep sequencing analysis (RNA-Seq) on the early T cells of NOD mice, just prior to the checkpoint violation, in comparison with cells from normal mice. Gene ontology and pathway analyses of differentially expressed genes indicate that NOD cells are significantly enriched in cell signaling genes. In addition, several genes associated with the DN2 stage are surprisingly highly expressed in the DN3 cells from the NOD background, suggesting laxity in the control of the transition through the DN2 to DN3 T-lineage commitment checkpoint. Differentially expressed genes that map to the QTL regions will be investigated further for their effects on TCR signaling and β-selection.

Publications

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Summary: The main focus of the research program is the analysis of the dorsal-ventral axis gene regulatory network (GRN) functioning in the early *Drosophila* embryo. In particular, we are interested in the morphogenetic movements that are controlled by this network during gastrulation. Our working hypothesis is that transcriptional inputs help to coordinate groups of cells during collective cell movements. Therefore, we believe that the analysis of *cis*-regulatory mechanism will impact our analyses of gastrulation cell movements. Our goal is to extend our imaging approaches to assay all the cells within a developing *Drosophila* embryo, in the context of defined genetic and molecular perturbation, in order to understand the movements of each and every cell as an output of the genomic regulatory code. In addition, we will strive to develop additional methods that will allow us to visualize transcription and the activation of signaling pathways in a live developing embryo. To describe development of an organism as a sequence of molecular events is our ultimate goal.

One unifying goal of the studies being conducted in my laboratory is to understand *how* genes are orchestrated to control development. To date, the basis for our studies relates to a GRN, which describes what is known of dorsal-ventral patterning in the early *Drosophila* embryo. Within this GRN, information regarding genetic interactions and *cis*-regulatory control of ~60 genes is detailed. These genes interact to specify patterning along the *Drosophila* dorsal-ventral axis, to control cell movements that drive gastrulation, as well as to influence the subsequent differentiation of cells into different tissue types. We have used this extensive knowledge base to provide mechanistic insights into the development of embryos: (1) to understand how genes are expressed with proper spatial precision to pattern the embryo; and (2) to define the functions of these differentially expressed genes in controlling morphogenesis and differentiation.

Cis-regulatory design: dynamic interpretation of transcription factor levels

We have assembled a provisional network that describes how ~60 genes interact during gastrulation to specify dorsal-ventral patterning and subsequently to control differentiation of cells in *Drosophila*. However, even after such an extensive analysis, it remained unclear how the transcription factor Dorsal can regulate the expression of genes in a broad lateral domain. By analysis of fixed samples, we found that there is not a clear correlation between levels of Dorsal and patterns of gene expression in dorsolateral regions of the embryo (Liberman *et al*., *PNAS* 2009). We have conducted an analysis of the *cis*-regulatory sequences supporting expression in the broad lateral domain of embryos, and found evidence that cooperation between Dorsal and a ubiquitous activator is required to support expression in a broad lateral domain (Liberman and Stathopoulos, *Dev. Biol*., 2009). Our approach combined evolutionary analysis, site-directed mutagenesis, and synthetic construct design to support this model. Furthermore, we provide evidence for flexibility in the composition and organization of sites required to support expression within this domain.

ChIP-chip and ChIP-seq analyses will be analyzed to identify *cis*-regulatory sequences, not yet identified, for genes that are differentially expressed along the dorsal-ventral axis (Ozdemir *et al*., in review). Our prediction would be that different examples of *cis*-regulatory design will be identified, which would explain why we had not been able to identify these regions in the past using standard bioinformatic methods and our prior knowledge. In fact, our ChIP-seq analyses have identified new *cis*-regulatory modules that control expression of genes in the early embryo, which were not appreciated beforehand and provide new insights into the underlying embryonic patterning process. Using BAC recombineering technology, we are studying the interactions of *cis*-regulatory modules and have uncovered new insights including that enhancer-promoter interactions are regulated temporally, as well as that repressors are shared between *cis*-regulatory modules (Dunipace *et al*., in preparation).

Furthermore, 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. We are currently using genetic and computational approaches to understand how patterning is controlled by morphogen gradients in the wing disc (Nahmad and Stathopoulos, *PLoS Biol.*, 2009), as well as the embryo (Nahmad *et al*., in preparation).

Cell movement coordination during migration of cells: high-level spatial organization

The function of many genes differentially expressed along the dorsal-ventral axis of *Drosophila* embryos is to coordinate the cell movements that are driving gastrulation. We are analyzing the mechanism by which mesoderm spreading is accomplished. With technical

advances in imaging and novel quantitative analyses, we have shown that mesoderm migration is a directed process; that cells move from ventral-most to dorsal-most regions of the ectoderm in a coordinate fashion (McMahon *et al*., *Science*, 2008). High-level spatial organization within the moving population of cells was visualized. Cells at the leading edge originate from a particular position within the invaginated mesoderm; cell divisions are regulated temporally and spatially; and intercalation events contribute to monolayer formation of the migrating collective. We aim to determine whether such spatial organization is required for collective cell migration in general, a process that makes essential contributions to embryonic development.

To this aim, we are extending our cell tracking analyses to a different group of cells that undergo a long-range migration during *Drosophila* embryogenesis (CVM: caudal visceral mesoderm cells), precursors of the longitudinal muscles that ensheath the larval gut. Genetic analysis coupled with live imaging and tracking has demonstrated that these cells undergo a directed migration that requires FGF signaling (Kadam *et al*., in preparation).

We also investigate FGF signaling mechanisms, in general, using *Drosophila* as a model system. Vertebrates have ~130 FGF ligand-receptor interactions, whereas, we have shown that in *Drosophila* there are only three functional combinations. We demonstrate that the FGF-8 homologous proteins, Pyramus and Thisbe, are not redundantly functioning ligands but instead these genes have distinct functions, due in part to differential range of action and in part to differential expression (Kadam *et al*., *Development*, 2009). In addition, our work has suggested that FGF signaling is important for collective cell mesoderm migration during gastrulation, but that it is not absolutely required. In the absence of FGF signaling, those cells in contact with the ectoderm are competent to migrate in a directional manner; those that cannot contact the ectoderm exhibit random movements, and lose the ability to move directionally. Loss of particular cell adhesion molecules (integrin beta-PS) exhibits a mesoderm migration defect similar to FGF mutants (McMahon *et al*., *Development* 2010). We hypothesize that FGF signaling may differentially regulate cell adhesion properties to influence cell migration.

In the future, we will determine if these ligands differ in range of action directly. To start, we have characterized the protein profiles for each protein and shown that Pyramus and Thisbe are cleaved to different sizes (Tulin and Stathopoulos, *BMC Dev. Biol.* 2010). We hypothesize regulated proteolytic cleavage may affect the ligands' signaling range.

106. Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in *Drosophila*

Louisa M. Liberman[*]*, Gregory T. Reeves*[*]*, Angelike Stathopoulos*

The NF-κB related transcription factor, Dorsal, forms a nuclear concentration gradient in the early *Drosophila* embryo patterning the dorsal-ventral axis to specify mesoderm, neurogenic ectoderm and dorsal ectoderm cell fates. These patterning events are thought to be determined by the concentration of nuclear Dorsal; however, the actual levels of nuclear Dorsal have not been quantified. Furthermore, existing models for Dorsal-dependent germ layer specification and patterning consider steady-state levels of Dorsal relative to target gene expression patterns, yet Dorsal gradient formation is dynamic as is gene expression. We devised a quantitative imaging method to characterize the dynamics of Dorsal nuclear gradient formation while simultaneously examining Dorsal target gene expression in nuclei along the dorsal-ventral axis. Unlike what has been observed in other insects such as Tribolium, we find that the Dorsal gradient maintains a constant bell-shaped distribution during embryogenesis. We also find that some genes that require Dorsal for activation fall outside the graded localization of Dorsal, raising the question whether these genes are direct Dorsal targets. Additionally, we show that Dorsal levels change in time during embryogenesis such that steady state is not reached even at cellularization. These results suggest that the multiple gene expression outputs observed along the dorsal-ventral axis do not simply reflect a steady-state Dorsal nuclear gradient. Instead we propose that the Dorsal gradient supplies positional information throughout nuclear cycles 10 through 14 and that compensatory combinatorial interactions between Dorsal and other factors effect differential gene expression along the dorsal-ventral axis.

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Publication

Liberman, L.M., Reeves, G.T. and Stathopoulos, A. (2009) Quantitative imaging of the Dorsal nuclear gradient reveals limitations to threshold-dependent patterning in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **106**(52):22317-22322.

107. Design flexibility in *cis*-regulatory control of gene expression

Louisa Liberman, Angelike Stathopoulos

In early *Drosophila* embryos, the transcription factor Dorsal regulates patterns of gene expression and cell fate specification along the dorsal–ventral axis. How gene expression is produced within the broad lateral domain of the presumptive neurogenic ectoderm is not understood. To investigate transcriptional control during neurogenic ectoderm specification, we examined divergence and function of an embryonic *cis*-regulatory element controlling the gene short gastrulation (sog). While transcription factor binding sites are not completely

conserved, we demonstrate that these sequences are bona fide regulatory elements, despite variable regulatory architecture. Mutation of conserved sequences revealed that putative transcription factor binding sites for Dorsal and Zelda, a ubiquitous maternal transcription factor, are required for proper sog expression. When Zelda and Dorsal sites are paired in a synthetic regulatory element, broad lateral expression results. However, synthetic regulatory elements that contain Dorsal and an additional activator also drive expression throughout the neurogenic ectoderm. Our results suggest that interaction between Dorsal and Zelda drives expression within the presumptive neurogenic ectoderm, but they also demonstrate that regulatory architecture directing expression in this domain is flexible. We propose a model for neurogenic ectoderm specification in which gene regulation occurs at the intersection of temporal and spatial transcription factor inputs.

Publication

Liberman, L.M. and Stathopoulos, A. (2009) Design flexibility in *cis*-regulatory control of spatial gene expression: synthetic and comparative evidence. *Dev. Biol.* **327**(2):578-589.

108. Live imaging studies of the Dorsal transcription factor nuclear gradient

Gregory T. Reeves, Angelike Stathopoulos

A nuclear gradient of the transcription factor Dorsal is responsible for patterning the entire dorsal-ventral axis in early *Drosophila* development. However, questions still remain regarding the spatial and temporal behavior of this gradient. In our previous study (Liberman *et al.*, 2009), we quantified the Dorsal nuclear gradient in fixed tissue, concluding that on average, its amplitude increases throughout nuclear cycles 10-14, while its spatial extent remains roughly constant from nuclear cycle to nuclear cycle. However, our results left open the question of how the tails of the gradient behave.

In this project, we are addressing two questions: (1) what is the behavior of the tails of the gradient, and (2) what are the temporal dynamics of the gradient? To answer the first question, we are using an optimized protocol for the quantification of the gradient in fixed tissues. This optimized protocol allows us to definitively measure non-zero levels of nuclear Dorsal on the dorsal side of the embryo (which we call "basal levels").

In addition, we are using a BAC-recombineered Dorsal-GFP that complements the null mutant to support live imaging studies (Figure 1). Previous live studies of this system by another group used a Dorsal-GFP fusion that fails to complement the mutant and has critical sequences deleted from the *dorsal* portion of the transgene. Using our new Dorsal-GFP construct, our preliminary results show that the spatial extent of the gradient remains perfectly constant both from nuclear cycle to nuclear cycle (agreeing with the our previous study, Liberman *et al.*, 2009; red trace in Figure 2) but also *within* each nuclear cycle. We also conclude that the gradient amplitude grows over time within each nuclear cycle (blue trace in Figure 2), and the average amplitude of the gradient increases from nc 10-14. These results were obtained by imaging vertically-mounted embryos using standard confocal microscopy. We are currently working to image Dorsal-GFP embryos using lightsheet microscopy for better time and spatial resolution, higher signal-to-noise, and also lower phototoxicity, which may facilitate quantitation of Dorsal levels at the tails of the gradient.

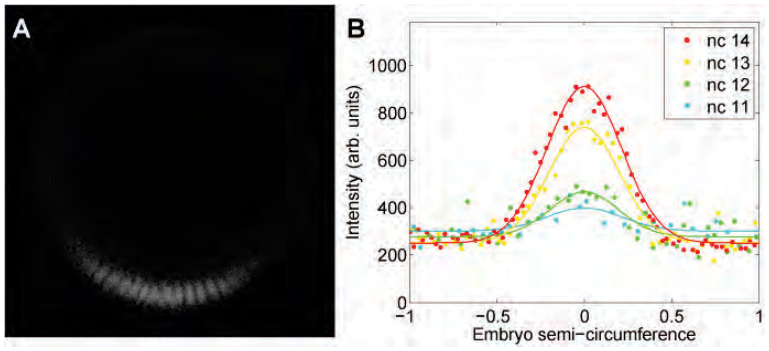

Figure 1: **(A)** Image of embryo expressing Dorsal-GFP. **(B)** Measurement of Dorsal-GFP across nc 11-14.

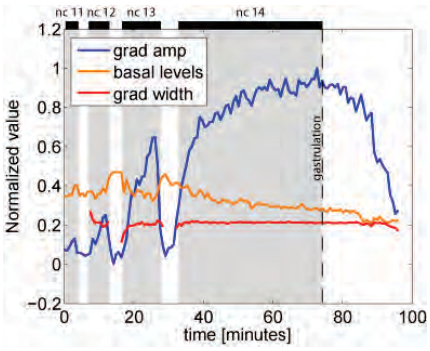

Figure 2: Gradient properties over time. Blue: amplitude, orange: basal levels, red: widths

109. High resolution mapping of Twist to DNA in *Drosophila* embryos: Efficient functional analysis and evolutionary conservation

Anil Ozdemir[*], Katherine Fisher[*], Shirley Pepke[1], Manoj Samanta[2], Leslie Dunipace, Kenneth McCue, Lucy Zeng[3], Nobuo Ogawa[3], Barbara Wold[4], Angelike Stathopoulos

Cis-regulatory modules (CRMs) function by binding sequence specific transcription factors, but the relationship between *in vivo* physical binding and the regulatory capacity of factor-bound DNA elements remains uncertain. We investigate this relationship for the well-studied Twist factor in *Drosophila melanogaster* embryos by analyzing genome-wide factor binding and testing the functional significance of Twist occupied regions and motifs within regions. Twist ChIP-seq data efficiently identified previously studied Twist-dependent CRMs and robustly predicted new CRM activity in transgenesis, with newly identified Twist-occupied regions supporting diverse spatiotemporal patterns (>75% positive, n =32). Some, but not all, candidate CRMs requires Twist for proper expression in the embryo. We also found the Twist motifs most favored in genome ChIP data (*in vivo*) differed from those most favored by SELEX (*in vitro*). The majority of ChIP-seq binding could be parsimoniously explained by a CABVTG motif located within 50bp of the computationally identified ChIP-seq summit, and of these E-boxes CACATG was most prevalent. Mutagenesis experiments demonstrated that these different Twist E-box motif types are not fully interchangeable, suggesting that the consensus ChIP-Seq derived consensus (CABVTG) combines sites having distinct regulatory outputs. Further analysis of position, frequency of occurrence, and sequence conservation for transcription factor motifs revealed a statistically significant enrichment and increased conservation of CABVTG E-box motifs near Twist ChIP-Seq signal summits, a region of approximately ±150bp surrounding summits of Twist binding that is preferentially conserved, and a very general depletion of A/T-rich sequences from input chromatin that extends to ChIP-Seq data from multiple genomes and laboratories. Our results show that high resolution *in vivo* binding data can be the basis for diverse functional experiments needed to provide insights into global and local *cis*-regulatory logic and to probe the relationship between *in vivo* factor occupancy and regulatory action.

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110. Using ChIP-seq and BAC recombineering approaches to study enhancer-promoter interactions

Leslie Dunipace, Anil Ozdemir, Angelike Stathopoulos

In a previous study, we conducted an analysis of Twist binding in the genome using ChIP-seq (Ozdemir *et al*., in review). Twist is a bHLH transcription factor that influences patterning of the dorsal-ventral (DV) axis of *Drosophila* embryos. In addition to binding at bona fide *cis*-regulatory modules (CRMs) that support expression along the DV axis of the embryo, we found that binding of Twist is observed with high frequency at promoter proximal positions that are not able to support expression in isolation. For example, Twist binds to three distinct regions at the *brinker* (*brk*) locus: to two distinct CRMs thought to support "overlapping expression" (one upstream and the other downstream of the gene), as well as to the *brk* promoter. To assay the function of these regions of binding, we employed recombineering methods to manipulate large DNA fragments containing the *brk* locus. First, we inserted the GFP into the *brk* first exon creating a reporter gene that allows assay of expression in the native environment of the gene. Next, we deleted each of the three regions of Twist binding and assayed the effect on GFP reporter expression. In this way, we demonstrate that enhancers for the *brinker* gene function autonomously within the early embryo to support expression and importantly that expression supported is both spatially and temporally distinct. Furthermore, we show that the promoter proximal binding detected supports long-range enhancer-promoter interactions. We are investigating how the promoter proximal region may facilitate the switch from the early enhancer to the second enhancer, which functions at a later timepoint.

111. *Cis*-regulatory analysis of *snail* demonstrates the distinct functions of two early embryonic enhancers and their interaction, including dominant repression by the distal enhancer

Leslie Dunipace, Anil Ozdemir, Angelike Stathopoulos

Whole-genome methods have greatly improved our understanding of how *cis*-regulatory sequences contribute to animal development. High throughput sequencing of chromatin-immunoprecipitations (ChIP-seq) can identify the DNA sequences occupied *in vivo* by transcription factors to within 50 bp of a binding site, and BAC recombineering methods coupled with efficient site-directed integration of large transgenes into the *Drosophila* genome facilitate the assay of *cis*-regulatory modules in the context of the native gene locus. Here we show how these techniques can be used to provide insights into *cis*-regulatory mechanisms. We analyzed the significance of identified Twist binding to the *snail* locus. *snail* encodes a Zinc-finger transcriptional repressor that is influential for delineating the mesoderm vs. mesectoderm/neurogenic-ectoderm boundary in the embryo. In 1991, Ip *et al*., showed that the ~2 kB sequence upstream of the *snail* gene

can support expression comparable to the endogenous pattern when assayed in a standard reporter gene assay. However, we noticed that the pattern supported was not as sharp as the endogenous gene. The Twist ChIP-seq experiment identified binding to both *snail* promoter proximal regions (i.e., the Ip enhancer), as well as to a region greater than 10 kB upstream of the gene, within the intron of a flanking gene. We show that this region functions to support expression of *snail* that is comparable to the endogenous expression pattern, and is also as sharp as the wildtype pattern. The distal enhancer contains sites for the repressor Huckebein, and presumably by looping interactions this repressor can function in a dominant fashion to silence the promoter proximal enhancer. By BAC recombineering approach, we show that the distal enhancer is required to support the function of Snail, whereas, the proximal enhancer is dispensable. Therefore, reporter gene analyses conducted in the context of the native gene locus demonstrate that two enhancers support *snail* expression in the early embryo, that they are not redundant, and that the distal enhancer contains repressor activity that is shared as it functions to silence the promoter proximal enhancer.

112. Repressors define both the dorsal and ventral borders of Dorsal target genes

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, by activating the expression of tissue-specific genes along the dorsal-ventral axis. Current models postulate that limiting amounts of Dorsal establish the dorsal boundaries of gene expression. In the case of the gene *intermediate neuroblast defective (ind),* it is believed activation mediated by Egfr signaling contributes to the pattern and helps to establish the dorsal border. We have evidence suggesting that instead repressors are necessary to define the sharp dorsal boundary of the *ind* pattern. Previous synthetic enhancer analysis of the *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 these 12 base pairs alone are sufficient to mediate repression in dorsal regions. Furthermore, when this element is mutated within the full-length *ind* enhancer, expression is expanded dorsally demonstrating this element is required to support repression in dorsal regions. We identified putative repressors using affinity chromatography and mass spectrometry. Our data suggests *ind* expression mediated by Dorsal and Egfr signaling is broad and is refined by repressors. The broader implication of our analysis is that repressors function not only in ventral regions but also in dorsal regions of the embryo to support patterning of the dorsal-ventral axis. Therefore, mechanisms used to regulate gene expression along the anterior-posterior and dorsal-ventral axes may be more similar than previously thought.

113. Dynamic interpretation of hedgehog signaling in the *Drosophila* wing disc

Marcos Nahmad, Angelike Stathopoulos

Morphogens are classically defined as molecules that control patterning by acting at a distance to regulate gene expression in a concentration-dependent manner. In the *Drosophila* wing imaginal disc, secreted Hedgehog (Hh) forms an extracellular gradient that organizes patterning along the anterior-posterior axis and specifies at least three different domains of gene expression. Although the prevailing view is that Hh functions in the *Drosophila* wing disc as a classical morphogen, a direct correspondence between the borders of these patterns and Hh concentration thresholds has not been demonstrated. Here, we provide evidence that the interpretation of Hh signaling depends on the history of exposure to Hh and propose that a single concentration threshold is sufficient to support multiple outputs. Using mathematical modeling, we predict that at steady state, only two domains can be defined in response to Hh, suggesting that the boundaries of two or more gene expression patterns cannot be specified by a static Hh gradient. Computer simulations suggest that a spatial "overshoot" of the Hh gradient occurs, i.e., a transient state in which the Hh profile is expanded compared to the Hh steady-state gradient. Through a temporal examination of Hh target gene expression, we observe that the patterns initially expand anteriorly and then refine, providing *in vivo* evidence for the overshoot. The Hh gene network architecture suggests this overshoot results from the Hh-dependent up-regulation of the receptor, Patched (Ptc). In fact, when the network structure was altered such that the ptc gene is no longer upregulated in response to Hh-signaling activation, we found that the patterns of gene expression, which have distinct borders in wild-type discs, now overlap. Our results support a model in which Hh gradient dynamics, resulting from Ptc up-regulation, play an instructional role in the establishment of patterns of gene expression.

Publication

Nahmad, M. and Stathopoulos, A. (2009) Dynamic interpretation of hedgehog signaling in the *Drosophila* wing disc. *PLoS Biology* **7**(9):e1000202.

114. Establishing positional information through gradient dynamics

Marcos Nahmad, Angelike Stathopoulos

A longstanding question in developmental biology is how morphogen gradients establish positional information during development. Although the existence of gradients and their role in developmental patterning is no longer in doubt, the ability of cells to respond to different morphogen concentrations has been controversial. In the *Drosophila* wing disc, Hedgehog (Hh) forms a concentration gradient along the anterior-posterior axis and establishes at least three different gene expression patterns. In a recent study, we challenged the prevailing idea that Hh establishes positional information in a dose-dependent manner and proposed a model in which dynamics of the

gradient, resulting from the Hh gene network architecture, determines pattern formation in the wing disc. In this extra view, we discuss further the methodology used in this study, highlight differences between this and other models of developmental patterning, and also present some questions that remain to be answered in this system.

Publication

Nahmad, M. and Stathopoulos, A. (2010) Establishing positional information through gradient dynamics: A lesson through the hedgehog-signaling pathway. *Fly* **4**(4):1-5.

115. Probing the role of morphogen gradient dynamics in developmental patterning

Marcos Nahmad

Morphogen-mediated patterning is the predominant mechanism by which positional information is established during animal development. In the classical view, the interpretation of morphogen gradients is assumed to be at equilibrium and the dynamics of gradient formation are generally ignored. The problem of whether or not morphogen gradient dynamics contribute to developmental patterning has not been explored in detail, in part, because genetic experiments that selectively affect signaling dynamics while maintaining unchanged the steady-state morphogen profile are difficult to design and interpret. Here, I present a theoretical approach to identify genetic mutations in developmental patterning that may affect the transient, but leave invariant the steady-state signalling gradient. As a case study, I illustrate how these tools can be used to explore the dynamic properties of Hedgehog signaling in the developing wing of the fruit fly, *Drosophila melanogaster*. This analysis provides insights into how different properties of the Hedgehog gradient dynamics, such as the duration of exposure to the signal or the width of the gradient prior to reaching the equilibrium, can be genetically perturbed without affecting the local steady-state distribution of the gradient. I propose that this method can be generally applicable as a tool to design experiments to probe the role of transient morphogen gradients in developmental patterning and discuss potential applications of these ideas to a wide variety of problems.

116. Quantitative imaging of collective cell migration during *Drosophila* gastrulation: multiphoton microscopy and computational analysis

Willy Supatto[1], Amy McMahon, Scott E. Fraser[2], Angelike Stathopoulos

This protocol describes imaging and computational tools to collect and analyze live imaging data of embryonic cell migration. Our five-step protocol requires a few weeks to move through embryo preparation and four-dimensional (4D) live imaging using multiphoton microscopy, to 3D cell-tracking using image processing, registration of tracking data, and their quantitative analysis using computational tools. It uses commercially available equipment, and requires expertise in microscopy and programming that is appropriate for a biology laboratory. Custom-made scripts are provided, as well as sample datasets to permit readers without experimental data to perform the analysis. The protocol has offered new insights into the genetic control of cell migration during *Drosophila* gastrulation. With simple changes, this systematic analysis could be applied to any developing system: the definition of cell positions in accordance with the body plan, the decomposition of complex 3D movements, and the quantification of the collective nature of cell migration.

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Publication

Supatto, W., McMahon, A., Fraser, S.E. and Stathopoulos, A. (2009) Quantitative imaging of collective cell migration during *Drosophila* gastrulation: multiphoton microscopy and computational analysis. *Nature Proto.* **4**(10):1397-1412.

117. Mesoderm migration in *Drosophila* is a multi-step process requiring FGF signaling and integrin activity

Amy McMahon, Gregory T. Reeves, Willy Supatto, Angelike Stathopoulos

Migration is a complex, dynamic process that has largely been studied using qualitative or static approaches. As technology has improved, we can now take quantitative approaches towards understanding cell migration using *in vivo* imaging and tracking analyses. In this manner, we have established a four-step model of mesoderm migration during *Drosophila* gastrulation: (I) mesodermal tube formation, (II) collapse of the mesoderm, (III) dorsal migration and spreading and (IV) monolayer formation. Our data provide evidence that these steps are temporally distinct and that each might require different chemical inputs. To support this, we analyzed the role of fibroblast growth factor (FGF) signaling, in particular the function of two *Drosophila* FGF ligands, Pyramus and Thisbe, during mesoderm migration. We determined that FGF signaling through both ligands controls movements in the radial direction. Thisbe is required for the initial collapse of the mesoderm onto the ectoderm, whereas, both Pyramus and Thisbe are required for monolayer formation. In addition, we uncovered that the GTPase Rap1 regulates radial movement of cells and localization of the beta-integrin subunit, Myospheroid, which is also required for monolayer formation. Our analyses suggest that distinct signals influence particular movements, as we found that FGF signaling is involved in controlling collapse and monolayer formation but not dorsal movement, whereas, integrins are required to support monolayer formation only and not earlier movements. Our work demonstrates that complex cell migration is not necessarily a fluid process, but suggests instead that different types of movements are directed by distinct inputs in a stepwise manner.

Publication

McMahon, A., Reeves, G., Supatto, W. and Stathopoulos, A. (2010) Mesoderm migration in *Drosophila* is a multi-step process requiring FGF signaling and integrin activity. *Development* **137**(13):2167-75.

118. Investigating the coordination of the migrating mesoderm collective

Nathanie Trisnadi, Angelike Stathopoulos

We study the mesoderm in *Drosophila* embryos that undergoes collective cell migration during gastrulation. The FGF pathway has long been known to be a required component for proper mesoderm development. However, its role was unclear until recently when work from our lab revealed that FGF is responsible for only a subset of mesoderm cells and that another signal is also functioning in mesoderm migration. We are currently investigating this unknown pathway(s). In addition, we are also interested to learn how cell membranes play a role in collective migration, specifically in protrusions for directional sensing and in maintaining cell-cell contacts through adhesion molecules. To address these interests, we have begun a screen that focuses on proteins either secreted or located at the cell membrane. This collection, provided by the Zinn lab here at Caltech, allows us to specifically test proteins involved in signal transduction and cell-cell contacts. Proteins identified from the screen are further characterized by various methods to determine their role in mesoderm migration. These analyses include live imaging and cell tracking, which were previously developed in our lab. We hope these efforts will provide new insights into organized, collective cell migration.

119. FGF ligands in *Drosophila* have distinct activities required to support cell migration and differentiation

Snehalata Kadam, Amy McMahon, Phoebe Tzou, Angelike Stathopoulos

Fibroblast growth factor (FGF) signaling controls a vast array of biological processes including cell differentiation and migration, wound healing and malignancy. In vertebrates, FGF signaling is complex, with over 100 predicted FGF ligand-receptor combinations. *Drosophila melanogaster* presents a simpler model system in which to study FGF signaling, with only three ligands and two FGF receptors (FGFRs) identified. Here we analyze the specificity of FGFR [Heartless (Htl) and Breathless (Btl)] activation by each of the FGF ligands [Pyramus (Pyr), Thisbe (Ths) and Branchless (Bnl)] in *Drosophila*. We confirm that both Pyr and Ths can activate Htl, and that only Bnl can activate Btl. To examine the role of each ligand in supporting activation of the Htl FGFR, we utilize genetic approaches that focus on the earliest stages of embryonic development. When pyr and ths are equivalently expressed using the Gal4 system, these ligands support qualitatively different FGFR signaling responses. Both Pyr and Ths function in a non-autonomous fashion to support mesoderm spreading during gastrulation, but Pyr exhibits a longer functional range. pyr and ths single mutants exhibit defects in mesoderm spreading during gastrulation, yet only pyr mutants exhibit severe defects in dorsal mesoderm specification. We demonstrate that the *Drosophila* FGFs have different activities and that cell migration and differentiation have different ligand requirements. Furthermore, these FGF ligands are not regulated solely by differential expression, but the sequences of these linked genes have evolved to serve different functions. We contend that inherent properties of FGF ligands make them suitable to support specific FGF-dependent processes, and that FGF ligands are not always interchangeable.

Publication

Kadam, S., McMahon, A., Tzou, P., Payne, S. and Stathopoulos, A. (2009) FGF ligands in *Drosophila* have distinct activities required to support cell migration and differentiation. *Development* **136**(5):739-747.

120. Extending the family table: Insights from beyond vertebrates into the regulation of embryonic development by FGFs

Sarah Tulin, Angelike Stathopoulos

Since the discovery of Fibroblast Growth Factors (FGFs) much focus has been placed on elucidating the roles for each vertebrate FGF ligand, receptor, and regulating molecules in the context of vertebrate development, human disorders and cancer. Studies in human, mouse, frog, chick, and zebrafish have made great contributions to our understanding of the role of FGFs in specific processes. However, in recent years, as more genomes are sequenced, information is becoming available from many non-vertebrate models and a more complete picture of the FGF superfamily as a whole is emerging. In some cases, less redundancy in these FGF signaling systems may allow for more mechanistic insights. Studies in sea anemones have highlighted how ancient FGF signaling is and helped provide insight into the evolution of the FGF gene family. Work in nematodes has shown that different splice forms can be used for functional specificity in invertebrate FGF signaling. Comparing FGFs between urochordates and vertebrates, as well as between different insect species reveals important clues into the process of gene loss, duplication and subfunctionalization of FGFs throughout evolution. Finally, comparing all members of the FGF ligand superfamily reveals variability in many properties, which may point to a feature of FGFs as being highly adaptable with regards to protein structure and signaling mechanism. Further studies on FGF signaling outside of vertebrates is likely to continue to complement work in vertebrates by contributing additional insights to the FGF field and providing unexpected information that could be used for medical applications.

In press

Tulin, S. and Stathopoulos, A. Extending the family table: insights from beyond vertebrates into the regulation of embryonic development by FGFs. *Birth Defects Research Part C: Embryo Today*.

121. Signaling pathways for cell migration during fly development

Young-Kyung Bae, Angelike Stathopoulos

Coordinated cell movements are key events during animal development. We are using the *Drosophila* embryo to identify genetic and signaling pathways that govern cell migration in early embryo development. Previous quantitative analyses on the trunk mesoderm migration demonstrate that fibroblast growth factors (FGF) are responsible for the initial collapse of the mesoderm tube and monolayer formation (McMahon *et al*., 2010; Kadam *et al*., 2009; McMahon *et al*., 2008). We are investigating additional signaling pathways that contribute to the trunk mesoderm migration. From a candidate gene approach using RNAi screen, we identified that c-Jun NH2-terminal kinase (JNK) plays a role in mesoderm migration. In an embryo with a reduced JNK activity the mesoderm forms multilayer, suggesting that JNK is required for monolayer formation at the last stage of mesoderm migration. We are currently conducting a genetic analysis on JNK signaling pathway components, along with quantitative live imaging of mutant embryos. The goal is to determine whether JNK signaling directly regulates mesoderm spreading and/or whether this pathway function indirectly through influencing FGF signaling.

122. FGF ligands provide chemo-attractive cues to maintain bilateral symmetrical of migrating CVM cells

Snehalata Kadam, Angelike Stathopoulos

Fibroblast growth factor (FGF) signaling is required during many developmental processes including cell migration, cell differentiation, and also to support cell survival. Here we analyze the role of FGFR [Heartless (Htl)] activation by each of the FGF ligands [Pyramus (Pyr) and Thisbe (Ths)] in the migrating caudal visceral mesoderm (CVM) cells, precursors of longitudinal muscles of the gut. First, we describe the migration of CVM cells using live *in vivo* imaging, which is the longest in all of embryogenesis. CVM cells emerge as a single cluster of ~40 cells in between the midgut and hindgut at the posterior tail of the embryo. They form two bilaterally symmetrical clusters at late stage 10 and start migrating anteriorly. They actively migrate by maintaining the bilateral symmetry from stage 11 until stage 13, during the same time as germ band retraction. Toward the end of migration the cells have positioned themselves to cover the gut from anterior to posterior. They then start differentiating by fusing with the fusion competent cells to result into multinucleate muscles forming the outer layer of the midgut. We show that both Ths and Pyr FGF ligands are required for migration and survival of CVM cell. The double *pyr* and ths mutants display migration defects. The formation of two clusters is defective, maintenance of bilateral symmetry is lost and toward the end of migration cells appear to be circular and dying. The FGF ligand Thisbe is required for specification of the two clusters and maintenance of synchrony of the bilateral CVM cell clusters. The FGF ligands Pyramus is required for maintenance of bilateral symmetry of the migrating CVM cells. Rescue experiments reveal Pyr and Ths provide chemoattractive cues to the CVM cells. Live Imaging of CVM cells in htl, double and single *pyr* and *ths* mutants reveals that CVM cells merge by crossing over the midline, there is a delay in migration and the cells dye toward the end of migration. The cell survival defects could be either direct due to lack of FGF signal or other indirect effect resulting from their veering off the migration track.

123. Characterization of the caudal visceral mesoderm migration in *Drosophila melanogaster* using ChIP-seq and cell tracking

Srimoyee Ghosh, Angelike Stathopoulos

The caudal visceral mesoderm (CVM) consists of a group of cells that migrate across the *Drosophila* body plan and ultimately give rise to the longitudinal muscles of the embryonic gut. Little is known about the genetic interactions necessary for this migration to occur and thus, far few genes have been characterized in playing a role in CVM migration. By performing ChIP-seq using two transcription factors present in CVM cells, we wish identify novel genes that are effecting the migration of these cells. Our lab has also discovered that discovered that mutations in the members of the FGF family (the receptor Heartless and its ligands, Pyramus and Thisbe) cause aberrant CVM migration. Through the use of tracking software, we wish to better characterize the FGF mutant phenotype in hopes of getting a better understanding of CVM migration, as well as clarifying the role FGF plays in this migration.

124. Analysis of Thisbe and Pyramus functional domains reveals evidence for cleavage of *Drosophila* FGFs

Sarah Tulin, Angelike Stathopoulos

As important regulators of developmental and adult processes in metazoans, Fibroblast Growth Factor (FGF) proteins are potent signaling molecules whose activities must be tightly regulated. FGFs are known to play diverse roles in many processes, including mesoderm induction, branching morphogenesis, organ formation, wound healing and malignant transformation; yet much more remains to be learned about the mechanisms of regulation used to control FGF activity. In this work, we conducted an analysis of the functional domains of two *Drosophila* proteins, Thisbe (Ths) and Pyramus (Pyr), which share homology with the FGF8 subfamily of ligands in vertebrates. Ths and Pyr proteins are secreted from *Drosophila* Schneider cells (S2) as smaller N-terminal fragments presumably as a result of intracellular proteolytic cleavage. Cleaved forms of Ths and Pyr can be detected in embryonic extracts as well. The FGF-domain is contained within the secreted ligand portion, and this domain alone is capable of functioning in the embryo when ectopically expressed. Through targeted ectopic expression experiments in which we assay the ability of full-length, truncated, and chimeric proteins to support cell differentiation, we find evidence that: (1) the C-terminal domain of Pyr is retained inside the cell and does not seem

to be required for receptor activation; and (2) the C-terminal domain of Ths is secreted and, while also not required for receptor activation, this domain does plays a role in limiting the activity of Ths when present. We propose that differential protein processing may account for the previously observed inequalities in signaling capabilities between Ths and Pyr. While the regulatory mechanisms are likely complex, studies such as ours conducted in a tractable model system may be able to provide insights into how ligand processing regulates growth factor activity.

Publication

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Tulin, S. and Stathopoulos, A. (2010) Extending the family table: Insights from beyond vertebrates into the regulation of embryonic development by FGFs. *Birth Defects Research Part C: Embryo Today: Reviews.* In press.

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Summary: We seek to understand how a genome controls development and behavior. We use *C. elegans* molecular genetics to understand detailed mechanisms, and functional genomics to obtain global views of development and behavior. 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.

Our efforts in genomics are experimental and computational. We worked with Caltech's Millard and Muriel Jacobs Genetics and Genome Laboratory to determine the genomic sequence of several nematode species using only short sequencing reads. One is an unnamed *Caenorhabditis* species (strain PS1010) that is an outgroup for the five existing sequenced species. We used cDNA sequence data to help assemble larger than gene-size pieces of this genome. By comparing the PS1010 genome to other *Caenorhabditis* species, we identified thousands of short, high conserved sequences that we hypothesis are regulatory. In addition, we have sequenced, assembled and annotated the genome of *Steinernema carpocapsae*, an insect-killing nematode that can jump onto hosts (see below). We are also helping to sequence the sheep parasite *Haemonchus contortus*.

Our behavioral studies focused this year on sexual attraction, sleep, and host finding by parasitic nematodes. We have continued to study the chemicals (ascarosides) that constitute mating pheromone made by hermaphrodites (morphologically females but that make sperm for internal self-fertilization) and sensed by males. We hypothesize that ascarosides are a diverse family of nematode signaling molecules. To test this hypothesis we are continuing our collaboration with the labs of Art Edison and Frank Schroeder to purify mating cues from other nematode species. To study parasite behaviors, we are using two genera of insect killing nematodes that are used in insect biocontrol because they deliver toxic bacteria to their hosts. One key discovery this year is that *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* use the same sensory neuron as *C. elegans* to respond to carbon dioxide. *Steinernema carpocapsae* is able to jump onto insects, and we are trying to understand the genetic and cellular basis for this amazing behavior, as well as its evolutionary origin, as only members of this genus can jump.

We have used channel rhodopsin to faithfully activate a neuron, as evidenced by whole-cell patch electrophysiology neuronal activity in a pre-synaptic cell expressing channel rhodopsin and then in its post-synaptic partner. Now that this system is validated, we are expressing channel rhodopsin and a genetically-encoded calcium sensor in a range of specific neurons to be able to examine neuronal circuit properties.

The infective juveniles (IJs) of *H. bacteriophora* and *S. carpocapsae* are analogous to the dauer larvae of *C. elegans*. Developing *C. elegans* larvae choose between proceeding directly to reproductive development or to arrest development as dauer larvae, depending on population density (signaled by several ascarosides) and the amount of food available. We are studying how larvae make this all-or-none decision. As worms exit the dauer stage they resume reproductive development and we have analyzed how the organization of genes into operon might facilitate a rapid transition to growth.

In the area of cell regulation, we have continued to study WNT and EGF signaling to define new components, how these two pathways interact, and what determines the specific outcomes of common signals. For this study we focus on the *C. elegans* vulva, a paradigm for analyzing organogenesis. In one project, we are using the polarity of the vulval secondary lineage to study how multiple types of WNT receptors act in concert or antagonistically. EGF controls development via the RAS/MAPkinase pathway and behavior via phospholipase C-gamma pathway. We had previously found that the EGF-receptor acts in a single neuron, ALA, to control a sleep-like state. We are testing other conserved signaling

pathways for common roles in sleep regulation. This year we discovered that a network of three homeobox-containing transcriptional regulatory proteins regulate expression of the EGF-receptor and other genes in the ALA neuron.

We are trying to learn how to efficiently define *cis*-regulatory elements using functional assays. We have established establishing pipelines for *cis*-regulatory computational analysis to define genomic elements that we test in transgenic *C. elegans*. For example, we tested some of our methods on elements that direct expression in the DVA neuron, which we had previously shown to control the extent of body flexion during locomotion. We are developing new assays for regulatory elements. For a number of projects, we want to identify all the genes that are expressed in a particular cell at a particular time. We thus are trying different methods of obtaining a transcriptional profile from a single cell; the male linker cell is our first test case.

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 other nematodes (www.wormbase.org). 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 *Mus musculus.* 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 that is used for analyzing genomic-scale experiments. We are developing automated and semi-automated methods to make extraction of information from papers more efficient. One of the applications is for associating genes with Gene Ontology annotations. Lastly, we are exploring ways of visualizing biological information.

125. Scaffolding a *de novo* genome of *Caenorhabditis* sp. 3 PS1010 with RNA-seq

Ali Mortazavi, Erich M. Schwarz, Brian Williams, Lorian Schaeffer, Igor Antoshechkin, Barbara J. Wold, Paul W. Sternberg

Nematode genomes range from 60-300 Mbp in size, 1/10 to 1/30th of a human genome. As a test case for *de novo* next-generation analysis of nematode genomes, we sequenced genomic DNA and mixed-stage cDNA of *Caenorhabditis* sp. 3 PS1010 using paired 75-nt reads from an Illumina GAII. *C.* sp. 3 PS1010 is an outgroup to *C. japonica* and the Elegans group (e.g., *C. elegans*, *C. briggsae*, *C. remanei*, and *C. brenneri*); analysis of the *C.* sp. 3 PS1010 genome should therefore define traits specific to the *Caenorhabditis* genus as a whole, rather than to an *elegans*-like subset of that genus. 230 million genomic reads yielded an 80 Mb assembly with an N50 of 5 kb, which covered 95% of previously assembled 429 kb contigs. We sequenced mixed-stage poly(A)$^+$ RNA with 47.3 million mappable 75-mers (including 5.1 million spliced reads); this separately assembled into 17.8 Mb of cDNA, with an N50 of 1.055 kb. Scaffolding genomic contigs with cDNA increased our genomic N50 to 9.4 kb, about the size of two genes in *Caenorhabditis*. We predicted 22,851 genes, detecting expression in ~80%. Multigenome alignment and data filtering identified 2,672 deeply conserved DNA sequences likely to encode regulatory elements or previously unknown ncRNAs. Among these 2.7K ultraconserved sequences, we found 23 motifs of 8-15 nt in length, which included both previously published regulatory motifs (muscle-specific, miRNA-specific, PHA-4-regulated, SLR-2-regulated, and Sp1-binding) and novel motifs associated with locomotion, reproduction, and larval development. Next-generation genomic and cDNA sequencing followed by their joint assembly is biologically useful strategy for genomic analysis.

126. A sensory code for host seeking in parasitic nematodes

Elissa Hallem, Adler Dillman

How do parasites encode information about host cues, and how are responses to different cues integrated to generate host-seeking behavior? We are addressing these questions using three species as models: *Heterorhabditis bacteriophora*, *Steinernema carpocapsae*, and *C. elegans.* We are comparing host-seeking behaviors in response to a large and diverse panel of ecologically-relevant odorants in parasitic infective juveniles (IJs) and *C. elegans* dauers. We find that host seeking involves a broad host signal (carbon dioxide), as well as a broad range of host odors. Overall, the parasitic nematodes respond more similarly to each other than to *C. elegans* despite their phylogenetic distance, supporting a key role for olfaction in their convergently evolved parasitic lifestyles. We showed previously that *C. elegans* adults avoid CO_2, and that this response requires the BAG neurons (Hallem and Sternberg, 2008). In contrast to adult *C. elegans,* we find that *C. elegans* dauers and parasitic IJs are robustly attracted to CO_2. BAG neurons are required for CO_2 attraction in all three species, as well as for attraction of *H. bacteriophora* to *Galleria mellonella* hosts. BAG neurons are also required for CO_2-evoked jumping in *S. carpocapsae.* Thus, BAG neurons contribute to multiple aspects of host seeking in parasitic worms. We are now examining the contribution of bacterial symbionts to host-seeking behaviors in parasitic nematodes.

127. Whole genomic sequencing of steinernematids illuminates mutualism and parasitism in nematodes

Adler R. Dillman, Ali Mortazavi, Igor Antoshechkin

Rapidly lethal endoparasitism of insects enhanced by obligate mutualism with Gram-negative enteric bacteria has arisen at least twice in Nematoda; within the Steinernematidae and Heterorhabditidae. Between these,

not only does *Steinernema* appear to be an older lineage, it is also more speciose. We utilize the robust phylogenetic framework and wealth of ecological data of *Steinernema* to inform the sequencing of eight whole nematode genomes (*S. carpocapsae, S. scapterisci, S. monticolum, S. siamkayai, S. intermedium, S. feltiae, S. riobrave,* and *S. glaseri*) along with their respective bacterial symbionts using the Solexa platform. Steinernematid genomes prove amenable to Solexa sequencing due to their size and high G+C content (~45%). Having the genomic sequence of eight closely related species will increase the resolution of comparative studies within *Steinernema* beyond that of any other nematode genus. We explore the utility of these genomes by examining the conservation of biological pathways (RNAi and dauer juvenile), and identify candidate genes involved in niche partitioning, host range, and mutualism within *Steinernema*.

128. Towards a SNP map for *Heterorhabditis bacteriophora*

Hillel Schwartz

Heterorhabditis bacteriophora is a species of insect-parasitic nematode that lives in mutually beneficial symbiosis with pathogenic bacteria of the genus *Photorhabdus*. *Photorhabdus* bacteria are lethal to insects and to other nematodes but are required for *H. bacteriophora* growth. The symbiosis between *H. bacteriophora* and *Photorhabdus* bacteria therefore offers the potential to study the molecular genetic basis of their cooperative relationship. We are developing tools to make such studies more feasible, and especially seek to develop tools and methods to make the nematode *H. bacteriophora* a more tractable system for molecular and genetic analysis. In particular, we are working on improved protocols for cryopreservation, mutagenesis, and transgenesis of *H. bacteriophora* and on the construction of a molecularly linked genetic map of *H. bacteriophora*. We have demonstrated that six independent isolates of *H. bacteriophora* are interfertile and are currently inbreeding them and characterizing their interactions with wild-type and mutant *Photorhabdus* bacteria. From these studies we will select an isolate or isolates and will use next-generation high-throughput sequencing technology for the purpose of refining the existing draft genome sequence and for the creation of a SNP map. We anticipate that this SNP map will enable us and the wider insect-parasitic nematode community to identify induced mutations and natural variations affecting the symbiotic interactions between *H. bacteriophora* nematodes and pathogenic *Photorhabdus* bacteria.

129. Next-generation genome and transcriptome of *Haemonchus contortus*

Erich M. Schwarz, Robin B. Gasser[*], Ali Mortazavi, Brian A. Williams, Igor Antoshechkin, Bronwyn E. Campbell[*], Neil D. Young[*], Aaron R. Jex[*], Cinzia Cantacessi[*], Ross Hall[*], Paul W. Sternberg

The Nematoda is one of the largest phyla in the animal kingdom and contains an enormous number of species with different life histories and adaptations. Although most nematodes are free-living, those that parasitize animals or plants cause serious diseases of major socioeconomic importance. A particularly important order is the Strongylida; one member in this order, *Haemonchus contortus* (barber's pole worm), which causes haemonchosis in small ruminants, has acquired drug resistance and has a substantial economic impact worldwide. In spite of this, little is known about the genetics and molecular biology of *H. contortus*. Sequencing the *H. contortus* genome and transcriptome will provide unique opportunities to explore its development, reproduction, parasite-host interactions and pathogenesis. This, in turn, should enable new methods of intervention and diagnosis. Sheer size and complexity of the *H. contortus* genome have hitherto been a challenge to its sequencing. To provide a serviceable *H. contortus* genome to the parasitology community, we have employed next-generation Illumina technology to draft the genome and sequence a developmentally-staged transcriptome. So far, we have produced a 318 Mb assembly with 5x coverage for the genome (N50 = 1.7 kb) and characterized the transcriptome of key developmental stages and both sexes of an Australian strain of the parasite. Transcriptomic data qualitatively improve the genomic data by aiding the prediction of protein-coding gene structures, enabling linkage of individual genomic contigs into scaffolds, allowing full open reading frames to be reliably predicted, and, most importantly, giving a digital readout of stage-specific and sex-specific patterns of gene expression. Currently, work is focused on enhanced assembly through the use of jumping libraries and transcript-mediated genomic assembly, and to analyze orthologies, motifs and stage-specific gene expression of its predicted proteome. We expect that these analyses, coupled with expression data, will support large-scale prediction of drug and vaccine targets for this important parasite and provide a resource to the community.

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130. WNT signaling and vulval lineage polarity

Paul Minor

The *C. elegans* vulva is formed from divisions of three vulval precursor cells (VPCs): P5.p, P6.p, and P7.p. P5.p and P7.p are induced and divide to form a secondary lineage pattern in which the daughter cells of P5.p and those of P7.p form a mirror image of each other. The orientation of these cells is established by the interaction of multiple Wnt signals: Wnts LIN-44 and MOM-2 act

through Fz/LIN-17 and Ryk/LIN-18, respectively, to promote the wild-type, anterior-facing P7.p, vulval lineage, termed refined polarity, whereas, Wnt EGL-20 acts through VANG-1 and Ror/CAM-1 to promote posterior-reversed vulval lineage (P-Rvl) of the P7.p daughter cells, termed ground polarity. Our data suggests a third protein LRP-2, a homolog of the mammalian Low density lipoprotein receptor related protein acts as a co-receptor of Wnt/EGL-20 along with receptors Ror/CAM-1 and VANG-1 to promote the P-Rvl phenotype. Initial site-of-action experiments do show *lrp-2* expression in the vulva as well. Furthermore, we have begun to unravel the molecular mechanisms by which the Wnt asymmetry pathway controls vulval polarity. We have shown that LRP-2 is essential for the correct localization of β-catenin/SYS-1, which ensures differential transcription of Wnt target genes between the daughter cells. Currently, we are focused on determining the binding mechanisms of LRP-2 and its physical interactions with Wnt and Ror proteins, as well as the protein domains necessary for correct vulval polarity through structure-function experiments. Another interesting question in this project concerns the molecular evolution of LRP proteins. Initial whole proteome analyses show that the LRP-2 protein is fairly specific to *Caenorhabditis*. The next step is to determine essential sequences/domains of this protein that lead to its function by comparing sequences across sequenced *Caenorhabditis* species.

131. A transcriptome of the migrating postembryonic *C. elegans* linker cell

Mihoko Kato, Erich Schwarz

Cell migrations are vital for the development of multicellular organisms. Because they are dynamically regulated, a broad understanding of a cell's migration requires characterization at different temporal stages. The *C. elegans* male linker cell (LC) is an individual cell that guides the complex yet highly stereotyped migration of the male gonad as the LC travels through much of the body length. We have previously demonstrated some stage-specific changes to the LC during the 3rd and 4th larval stages of migration and their regulation by *nhr-67*, an ortholog of *Drosophila tailless* (Kato and Sternberg, *Development*, 2009). For a more comprehensive understanding of LC migration, we have transcriptionally profiled the LCs of 3rd and 4th larval stage, wild-type males and 4th larval stage *nhr-67*(RNAi) males by microdissecting individual LCs, amplifying the mRNAs by RT-PCR, and performing ultra-high-throughput sequencing. Transcriptional profiling in the nematode *Caenorhabditis elegans* has so far been performed on large-scale harvests of stably differentiated embryonic or postembryonic cells, yet key events in development rely on individual cells that migrate and invade tissues dynamically. We have thus adapted laser microsurgery, patch-clamp pipettes, and 3'-tailed RT-PCR to enable RNAseq of individually dissected migrating linker cells from wild-type L3- and L4-stage male larvae, and from *nhr-67*(RNAi) L4 larvae. We have detected expression of 8,011 genes in wild-type L3 or L4 linker cells, versus 13,152 genes in bulk RNA from wild-type larvae (out of 20,252 genes in the *C. elegans* genome). 963 genes (~12% of 8,011) are robustly expressed in L3 or L4 linker cells (with an RPKM of at least 1), but have at least 20-fold lower expression in bulk larvae; this subset of linker-cell-specific genes includes *nhr-67*, along with over 53 other transcription factors such as the hormone receptor DAF-12, three HLH proteins, the Hox protein NOB-1, and the heterochronic zinc-finger protein LIN-29. We are currently assaying particularly interesting genes for function by GFP expression patterns. We have also observed 23 genes with strong linker cell phenotypes out of 200 screened. One of these genes, *srsx-18*, requires NHR-67 for its expression; *srsx-18*(RNAi) animals display slowed linker cell migration, which is a subset of the nhr-67(RNAi) phenotype, indicating that *srsx-18* represents a significant target of NHR-67 activation in linker cells. This analysis should provide the first transcriptional portrait of a migrating cell in *C. elegans*.

132. Cell type-specific profiling of the transcriptome in *C. elegans*

Meenakshi K. Doma, Igor Antoshechkin

Cell type-specific gene expression patterns underlie much of biology including development, physiology and behavior. Currently, analysis of cell-specific gene expression in *C. elegans* include strategies such as microarray analysis of RNA from surgically dissected tissue or sorting of GFP-tagged embryonic culture cells and the mRNA tagging technique used to profile specific tissues and cells. Each of these methods is limited in their applications. We are developing a new strategy to isolate RNA from specific cells that will enable cell type-specific transcriptome analysis in *C. elegans*. Recent studies have shown that uracil phosphoribosyltransferase (UPRT) from *Toxoplasma gondii* can be used for biosynthetic labeling of newly synthesized RNA from specific cells in multicellular organisms like *Drosophila*. We have adapted this method to *C. elegans* and designed transgenes that express TgUPRT in a spatially-restricted manner using cell type-specific promoters. Preliminary results show that on providing the uracil analog, 4-thiouracil (TU), it is modified and incorporated in newly synthesized RNA only from cells that express the TgUPRT. Our on-going and future efforts will be directed at purification of TU-tagged RNA followed by deep sequencing using RNA-seq in the context of specific biological questions. This method will provide *C. elegans* researchers a powerful tool that may provide novel insights into the regulation of gene expression at the single cell or tissue level.

133. A conserved signaling pathway promotes wakefulness in *C. elegans*

Julie Cho

The deleterious effects of dysfunctional sleep indicate that the process is tied to both human and animal health. Hence, it is essential to understand sleep regulation as well as the cellular and molecular pathways associated with the state. The quiescent state is conserved across a variety of both mammalian and non-mammalian species including the nematode, *Caenorhabditis elegans*. This state in worms is known as lethargus, and the state is behaviorally similar to sleep. Given that neurotransmitters and their neuronal receptors are high conserved between *C. elegans* and vertebrates, further studies in this simple system are expected to provide a relevant basis for modeling sleep in other organisms. Lethargus is characterized by the cessation of locomotor activity that characterizes the awake state in the wild-type worm. We used an automated tracking system to analyze locomotion of six mutations that increase neurotransmitter release. Four of these mutations increase cyclic AMP (cAMP). cAMP promotes wakefulness in *Drosophila*, and our findings show that increased cAMP signaling disrupts lethargus in worms. We will further study the site of action of these mutations.

134. Paired and LIM class homeodomain proteins coordinate differentiation of the *C. elegans* ALA neuron

Cheryl Van Buskirk

The ancient origin of sleep is evidenced by deeply conserved signaling pathways regulating sleep-like behavior, such as signaling through the Epidermal Growth Factor Receptor (EGFR). In *C. elegans*, a sleep-like state can be induced at any time during development or adulthood through conditional expression of LIN-3/EGF. The behavioral response to EGF is mediated by EGFR activity within a single cell, the ALA neuron, and mutations that impair ALA differentiation are expected to confer EGF-resistance and shed light on the mechanisms by which a single neuron adopts unique functional properties. We have identified three such EGF-resistant mutants. One of these corresponds to the LIM class homeodomain (HD) protein CEH-14/Lhx3, and the other two correspond to Paired-like HD proteins CEH-10/Chx10 and CEH-17/Phox2. While CEH-14 is required for ALA-specific gene expression throughout development, the Prd-like proteins display complementary temporal contributions to gene expression, with the requirement for CEH-10 decreasing as that of CEH-17 increases. We have found that CEH-17 participates in a positive autoregulatory loop with CEH-14 in ALA, and that CEH-10, in addition to its role in ALA differentiation, functions in the generation of the ALA neuron. Similar to CEH-17, CEH-10 is required for the posterior migration of the ALA axons, but CEH-14 appears to regulate an aspect of ALA axon outgrowth that is distinct from that of the Prd-like proteins. Our findings reveal partial modularity among the features of a neuronal differentiation program and their coordination by Prd and LIM class HD proteins.

135. Identification of DVA interneuron-specific regulatory sequences in *Caenorhabditis elegans*

Carmie Puckett Robinson[*]*, Erich M. Schwarz*

We identified by multi-species conservation regulatory regions within three genes that produced expression in the DVA neuron. These three regions ranged in size from 190 bp (*nmr-1*) and 195 bp (*twk-16*) to 322 bp (*fax-1*). However, only the *twk-16* conserved region produced restricted expression in the tail in DVA. *In silico* comparison of these regions identified a GA-rich motif. Mutation of this GA-rich motif demonstrated it was not necessary for DVA expression. Phylogenetic footprinting using four nematode species identified a conserved region of 53 bp within the first intron of the *twk-16* gene that demonstrated regions that both activate and repress expression in DVA and other neurons. There was a minimal core region of 8 bp necessary for expression in DVA and other neurons. This same 8-bp region was identified *in silico* by phylogenetic footprinting using seven nematode species, demonstrating the potential resolution of expanded multi-species phylogenetic comparisons. The restricted expression of the *twk-16* gene relies on a combination of sequence regions with both positive and negative effects on expression in DVA and other neurons. These results are consistent with the modular organization of the *twk-16* enhancer and the presence of additional unidentified regions within the *twk-16* enhancer, not identified by conservation, which both activate and repress expression in other neurons.

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136. Myogenic transcriptional network

Steven Kuntz, Barbara Wold

To learn about transcriptional regulatory network control of a complex process such as tissue development we used the embryonic muscle differentiation network of *Caenorhabditis elegans* as a model. Muscle development is a convenient target for such analyses due to its relative simplicity and homogeneity compounded with its resilience following mutation. *C. elegans* is experimentally approachable in targeting the primary myogenic factor HLH-1/MRF, which can be mutated to observe how the network attempts to function and compensate in its absence. Through the use of RNAi knock-downs we were able to increase the amount of muscle specified in the nematodes and thereby identify a set of genes preferentially expressed in muscle. By observing gene expression levels via RNA-seq in both wild-type and mutant animals, we were able to determine the impact of *hlh-1* binding, observed through anti-HLH-1 ChIP-seq, on gene expression levels throughout the genome. We find that while 878 genes, 216 genes with preferential expression in muscle, have significantly reduced expression in the absence of *hlh-1*/MRF, few

genes are turned off. Instead, several other transcription factors are actually upregulated in the *hlh-1* mutants. The effects of the mutation are dampened through multiple processes, including independent myogenic factors and upregulated compensatory factors.

137. Gene regulation by bidirectional promoters in *C. elegans*

Margaret Ho

Bidirectional gene promoters lie between adjacent genes that are transcribed from opposite strands of DNA. Recent studies have shown that this type of regulatory architecture represents more than 10% of the genes in the human genome, where the transcription start sites of bidirectional gene promoters are separated by less than 1000 base pairs. Gene expression patterns of many bidirectional gene transcripts in different metazoan species is often correlated, but many also exhibit anti-correlated expression. However, the mechanisms of gene regulation that occur for bidirectional gene promoters have not been extensively pursued. Whether the bidirectional gene promoters may share regulatory regions or prevent each other from activation in certain regulatory contexts is unclear. We have initiated a study of bidirectional gene promoters in *C. elegans*, which has a relatively well-annotated genome sequence, rapid and well-studied development, and for which there are many powerful genetic and molecular tools. We have built transgenic reporter gene assays with fluorescent reporter genes mCherry and GFP, in which we plan to test different candidate regulatory regions from the surrounding and intervening regions of bidirectional promoters as enhancers and negative-regulatory elements such as insulators or silencers using transgenic single-site insertion. Our aim is to gain a better understanding of gene regulatory mechanisms involved in the control of this common gene topology.

138. Metazoan operons accelerate transcription and recovery rates

Alon Zaslaver

Existing theories efficiently explain why operons are advantageous in prokaryotes, but their emergence in metazoans is still an enigma. Combining genomic meta-analysis, experiment and theory, we explain how operons could be adaptive during metazoan evolution. Focusing first on *C. elegans*, we showed that operon genes, typically consisted of growth genes, are significantly up-regulated during recovery from multiple growth arrested states, and that this expression pattern is anti-correlated to the expression pattern of non-operon genes. In addition, we found that transcriptional resources are initially limited during arrest recovery, and that recovering worms are extremely sensitive to any additional limitation in transcriptional resources. By clustering growth genes into operons, fewer promoters compete for limited transcriptional machinery, effectively increasing the concentration of transcriptional resources and accelerating growth during recovery. A simple mathematical model of transcription dynamics reveals how a moderate increase in transcriptional resources can lead to a substantial enhancement in transcription rate and recovery. We found evidence for this design principle in different nematodes (*e.g., Pristionchus pacificus* and *Brugia malayi*) as well as in the chordate *Ciona intestinalis*. As recovery from a growth arrested state into a fast growing state is a physiological feature shared by many metazoans, operons could evolve as an evolutionary solution to facilitate these processes.

139. A modular library of small-molecule signals regulates social behaviors in the nematode *Caenorhabditis elegans*

Jagan Srinivasan, Stephan H. von Reuss[*], Parag Mahanti[*], Frank C. Schroeder[*], Paul W. Sternberg

Small-molecule signaling plays an important role in the biology of *Caenorhabditis elegans*. We have previously shown that ascarosides, glycosides of the dideoxysugar ascarylose, regulate both development and behavior in *C. elegans* [1]. Using differential analysis of NMR spectra (DANS), we identified additional ascarosides in the *C. elegans* metabolome [2]. We found that the mating signal consists of a synergistic blend of three dauer-inducing ascarosides, ascr#2, ascr#3, and ascr#8 [2]. The ascarosides ascr#2, ascr#3 and ascr#8 carry partially overlapping information, as ascr#3 is more potent as a male attractant than ascr#8 and ascr#2, whereas ascr#2 is slightly more potent than ascr#3 in promoting dauer formation. Two types of neurons, the ASK neurons and the male-specific CEM neurons, are required for male attraction by ascr#3, whereas only CEM neurons are required for response to ascr#8. We have now identified a number of novel variants of ascarosides, including several indole derivatives, representing a highly modular library of signaling molecules. Biological testing of synthetic samples of the recently identified compounds revealed that femtomolar concentrations of these ascarosides act as components of a potent aggregation pheromone for both *C. elegans* hermaphrodites and males. We are currently testing neuronal and genetic requirements for the response to the newly discovered ascarosides.

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140. Hormonal signal amplification controls irreversible development commitment of *C. elegans* in response to environmental conditions

Oren Schaedel, Adam Antebi[*]

Many animals can choose between different developmental fates to maximizing fitness. Despite the complexity of environmental cues and life history, different developmental fates are executed in a robust fashion. The mechanisms that guarantee robust execution of a development choice in such environments remain unknown. The nematode *Caenorhabditis elegans* serves as a powerful model to examine this phenomenon because it has an advanced toolkit for cellular and genetic manipulations, and can adopt one of two developmental fates depending on environmental conditions. Nematodes grown in favorable conditions (sufficient food, low population density) develop into adults whereas, nematodes grown in unfavorable conditions (insufficient food, high population density) arrest development as a stress-resistant diapause form called dauer. The steroid hormone dafachronic acid (DA), product of DAF-9/cytochrome P450, directs development to adulthood by regulating the transcriptional activity of the nuclear hormone receptor DAF-12. The known role of DA suggests that it may be the molecular mediator of environmental condition effects on the developmental fate decision, although the mechanism is yet unknown. We hypothesize that information from the environment is integrated and reduced to a single cell non-autonomous environmental integrator, thereby, explaining the tight binary nature of the developmental fate decision. We propose a fate coordination mechanism in which production of a small amount of DA is amplified, locking in the adult fate. Using a combination of laser ablations and time series image analysis, we demonstrate that upon the decision to become an adult, the XXX neuroendocrine cells act as a source releasing DA. As a result, DAF-12-dependent expression of *daf-9* in the epidermis is amplified and propagated from anterior to posterior, dispersing high amounts of DA throughout the body. This dispersion of DA drives adult programs in the gonad, epidermis and vulva. Furthermore, we demonstrate that the XXX cells are not necessary for maintaining the adult fate after the signal amplification has started. This indicates that the epidermal amplification also confers the irreversibility of the decision by uncoupling the execution of the decision from the environmental integrator. We propose that this relay serves as a robust fate-locking mechanism to enforce an organism-wide binary decision, despite noisy and complex environmental cues.

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141. Transfer at a thermosensory synapse in *C. elegans*

Anusha Narayan, Gilles Laurent

C. elegans is a compact and attractive system for neural circuit analysis. An understanding of the functional dynamics of neural computation requires physiological analyses. We undertook the first characterization of transfer at a central synapse in *C. elegans* by combining optical stimulation of targeted neurons with electrophysiological recording techniques. We show that the synapse between AFD and AIY, the first stage in the thermotactic circuit, exhibits excitatory, tonic and graded release. We measured the linear range of the input-output curve and estimate the static gain at the synapse as 0.056 (<0.1). Release was frequency independent, showing no signs of facilitation or depression. Additionally, the response was unaltered in *unc-13* mutants severely defective in fast synaptic transmission. This leads us to believe that the signal from AFD to AIY is probably transmitted through neuropeptides using the dense-core vesicle pathway, and experiments are currently underway to test this hypothesis. Overall, the AFD-AIY synapse seems designed for robust and reliable transmission of a scaled-down temperature signal from AFD to AIY, enabling AIY to continuously monitor temperature information and integrate it with other incoming sensory information. The combination of optical stimulation tools with neural recording techniques is a powerful way to probe and analyze neural circuitry.

142. Comprehensive functional dynamics in the neural network of *C. elegans*

Alon Zaslaver

A major goal of systems neuroscience is to understand how information is processed by the neural network. In that respect, the neural network of *C. elegans* offers a unique opportunity: It contains ‘only’ 302 neurons and its connectivity was fully mapped. However, while the blueprint of the neural network has been in our hands for nearly 30 years, little is known about how information flows in the network. To address such fundamental questions we employ state of the art optogenetic tools (e.g., GCaMP, Channelrhodopsin) together with microfluidic devices. This system allows a comprehensive analysis of the functional dynamics in the neural network in a single-neuron resolution. In addition, we develop new systems to measure neural activity in freely moving animals. These setups will be valuable for a wide range of fields: from basic questions, such as robustness and efficiency in information processing, to more applicative fields, such as neural plasticity, learning and memory, aging and neurodegenerative diseases.

143. Worm phenotype ontology: Integrating phenotype data within and beyond the *C. elegans* community

Gary Schindelman, Jolene S. Fernandes, Carol Bastiani, Karen Yook

C. elegans is an important genetic model system for the study of basic biological and biomedical principles, largely, through the use of phenotype analysis. Because of the growth of *C. elegans* as a genetically tractable model organism and the development of large-scale analyses, there has been a significant increase of phenotype data that needs to be managed and made accessible to the research community. To do so, a standardized vocabulary is necessary to integrate phenotype data from diverse sources, permit integration with other data types and render the data in a computable form. We have therefore created a hierarchically structured controlled vocabulary of terms that can be used to standardize phenotype descriptions in *C. elegans*, namely the Worm Phenotype Ontology (WPO). The WPO comprises 1,880 phenotype terms, 74% of which are currently being used in the annotation of phenotypes associated with greater than 18,000 *C. elegans* genes. The scope of the WPO is not exclusively limited to *C. elegans* biology, rather it is devised to also incorporate phenotypes observed in related nematode species. Furthermore, we have enriched the value of the WPO by including cross-product ontologies, thereby increasing the accessibility of worm phenotypes to non-nematode biologists. This standardized phenotype ontology will therefore help to facilitate data retrieval, and cross-species comparisons within the nematode community as well as promote interoperability across the different Model Organism Databases (MODs).

144. Automatic categorization of diverse experimental information in the bioscience literature

Ruihua Fang, Gary Schindelman, Kimberly Van Auken, Jolene Fernandes, Wen Chen, Xiaodong Wang, Paul Davis[1], Mary Ann Tuli[1], Steven Marygold[2], Gillian Millburn[2], Beverley Matthews[3], Haiyan Zhang[3], William M. Gelbart[3], Nick Brown[4], Paul W. Sternberg

The phenomenal growth in bioscience literature has posed a great challenge in information retrieval both for general researchers and those whose task it is to extract information from the literature (biocuration) into public databases, which are invaluable resources for researchers. Due to the limitation of resources and manpower, it is increasingly difficult to keep curation information current. We developed an automatic method based on the machine learning method Support Vector Machine (SVM) for the first step practiced by many model organism databases (MODs) in the biocuration process—identifying papers containing specific types of data/information among published scientific papers. This step normally requires a curator to examine numerous papers to ascertain which few contain information of curation interest. Our classification system is completely automatic and can be readily applied to diverse data types. It is being used in the work flow at WormBase for automatic association of newly published papers with ten different data types including RNAi, antibody, phenotype analysis, gene regulation, mutant allele sequence, gene expression, gene product (GO), overexpression phenotype, gene interaction, and gene structure correction. We also successfully tested our method on fifteen different data types from FlyBase and three data types from Mouse Genomics Informatics (MGI). To have satisfactory performance, sufficient training papers are needed. Often a single database does not have enough training papers for some data types. We developed a simple, readily automated procedure of using training papers of similar data types from different databases such as WormBase and FlyBase to classify these data types for a single database. We believe that the work presented here can contribute greatly to the tremendous task of automating the important yet labor–intensive biocuration efforts.

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145. Semi-automated curation of gene ontology terms for WormBase using Textpresso, Support Vector Machines (SVMs), and Hidden Markov Models (HMMs)

Kimberly Van Auken, Ruihua Fang, Juancarlos Chan, Joshua Jaffery, Hans-Michael Müller

Manual curation of experimental data from the biomedical literature is an expensive and time-consuming endeavor. Nevertheless, most biological knowledge bases still rely heavily on manual curation for data extraction and entry. Text mining software and document classification algorithms that aid in information retrieval from the literature can provide a significant boost to manual curation efforts. We first employed the Textpresso category-based information retrieval and extraction system (http://www.textpresso.org), to explore how Textpresso might improve the efficiency with which WormBase curators manually curate *C. elegans* proteins to the Gene Ontology's Cellular Component Ontology. Using a training set of sentences that describe results of localization experiments in the published literature, we generated three new curation task-specific categories containing words and phrases associated with reports of experimentally determined subcellular localization. Using these categories to search the full text of articles we found that Textpresso searches identified curatable papers with recall and precision rates of 79.1% and 61.8%, respectively, and relevant sentences within those articles with recall and precision rates of 30.3% and 80.1%. From the identified sentences, curators were able to make 66.2%

of all possible experimentally supported GO Cellular Component annotations with 97.3% precision. To improve the precision of paper retrieval, we have now devised a combinatorial strategy in which the full text of incoming papers is first subject to an SVM-based classification scheme to filter potential false positive papers. This approach has been successful at filtering up to 75% of false positives documents. We are now expanding these semi-automated curation methods to two aspects of Gene Ontology Molecular Function curation: 1) macromolecular interactions and 2) enzymatic and transporter activities. We find that the combinatorial approach of SVM classification and Textpresso also works well for curation of macromolecular interactions, but is not yet optimal for enzymatic and transporter activities. To annotate these latter functions, we thus trained an HMM to identify candidate sentences for curation, with plans to incorporate a combined SVM and HMM approach to curating these data in the future.

146. Toward an interactive article: integrating journals and biological databases

Arun Rangarajan, Tim Schedl[1], Karen Yook, Juancarlos Chan, Stephen Haenel[2], Tracey DePellegrin-Connelly[3], Ruth Isaacson[3], Marek S. Skrzypek[4], J. Michael Cherry, Hans-Michael Müller

Journal articles and databases are two major modes of communication in the biological sciences hence, integrating these critical resources is of urgent importance to increase pace of discovery. Current entity recognition tools can only identify and link entities that are already present in a database, since they are fully automatic. Our pipeline incorporates inputs from authors and manual quality control (QC), which enhance the quality of the interactive articles. The pipeline described in this article uses entities in databases as a first step. Along with this, a web form is provided to authors to declare any new entities they have discovered/described in their article. These new entities in the article are also linked and incorporated into the database quickly. After entity linking, a manual quality control (QC) step is performed to resolve links for any ambiguous entities and correct any out-of-context links, thus improving the quality of the interactive article. The QC step also provides valuable feedback to improve the quality of the database. The interactive articles obtained through the described pipeline are data rich and of high quality as compared to articles processed with fully automated tools and provides important benefits to authors, readers and databases.

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147. The virtual worm: 3D renderings of *Caenorhabditis elegans*

Christian Grove

The organization of biological data into concise, intuitive, and engaging modes of communication will no doubt become an ever increasingly important goal of modern biological and medical sciences. Certainly, visual aids and 3-dimensional computer models will provide useful platforms for studying, as well as annotating, consolidating and exploring large amounts of otherwise unwieldy data. We developed a preliminary 3D visual computer model of the free living nematode (roundworm) *Caenorhabditis elegans* developed using the open source, Python-based 3D rendering program, Blender. The model is a to-scale rendering of an adult hermaphroditic worm, representing all of the major body tissue types including the skin, muscles, intestine, germline, and nervous system at the resolution of individual cells. *C. elegans* is an extremely useful model system in which to initiate the development of high-resolution visual models (from cells to molecules) because it has a fixed number of somatic cells (959 somatic nuclei), an invariable cell lineage, a completely mapped neural network consisting of 302 neurons, and comprehensive archives of transmission electron micrographs revealing the location, structure, and quality of subcellular organelles throughout the worm. We hope this model provides a framework for detailed annotation and an intuitive graphical user interface to allow exploration of the cellular and molecular biology of *C. elegans*.

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Structural, Molecular and Cell Biology

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Summary:

a. Small RNAs as determinants of epigenetic states in germline

Gene silencing via the RNA interference (RNAi) pathway is an evolutionary conserved process that is critical for the control of gene expression in organisms ranging from yeast to humans. Targets of RNAi are recognized through complementary base-pairing interactions with small RNAs that act as guides to RNAi effector complexes. Several distinct classes of endogenous small RNAs regulate gene expression states to impact diverse biological processes. Our lab focuses on understanding the nature and biological functions of small RNA pathways in animals.

We have identified and characterized an evolutionary conserved small RNA pathway that operates in germ cells and that is critical both for germline stem cell maintenance and for gametogenesis. Working in Drosophila and mice, we discovered a new class of small RNAs, Piwi-interacting (pi)RNAs. Piwi/piRNA pathway plays an important role in genome integrity by repressing selfish repetitive elements. A characterization of piRNA sequences in combination with genetic studies revealed that the biogenesis and function of piRNAs differs from that of other classes of small RNAs. While canonical small RNAs, such as microRNAs, affect gene expression post-transcriptionally, our studies suggest that piRNAs most likely serve as guides for *de novo* DNA methylation in mouse male germ cells.

We are interested in two general questions: biogenesis and function of small non-coding RNAs.

b. Biogenesis of piRNA[*]

Processing of piRNAs differs from that of other known classes of small RNAs. It was shown piRNA are produced independently of Dicer, the nuclease that generates siRNAs and microRNAs from double-stranded substrates; however, the proteins that are responsible for producing piRNAs are only partially understood.

Our investigations of piRNA biogenesis led us to the ping-pong model that proposes amplification of piRNAs in a cycle that depends on the nuclease activity of Piwi proteins themselves. One of the central mysteries of repeat silencing in both mammals and flies is how repeats are distinguished from genes and selectively silenced. We are investigating the nature of the determinants that make a particular sequence a target of the Piwi pathway. We are using biochemical purification of Piwi-piRNA complexes and genetic approaches to identify proteins involved in piRNA biogenesis.

c. Functions of the Piwi pathway and piRNA-guided de novo DNA methylation

We showed that the piRNA pathway is linked to *de novo* DNA methylation in the mouse germline. One of the three murine Piwi proteins is specifically found in germ cell nuclei during the critical window when *de novo* methylation patterns are established. We also showed that Piwi proteins at that developmental timepoint are associated with piRNAs that target several classes of transposable elements. The same transposons are de-repressed and their genomic sequences lose methylation in Piwi-deficient mice. The discovery that piRNAs may guide DNA methylation in germ cells is an important finding for several reasons. First, it provides a new paradigm for how small RNAs can affect gene expression. Second, it explains how a subset-of-sequences are tagged for *de novo* methylation. How methylation sites are defined remains a central mystery of epigenetics. An important goal of my lab is to define the pathway by which piRNAs guide *de novo* DNA methylation. We also study whether the piRNA pathway can be re-programmed to new targets and can be used to manipulate DNA methylation patterns in somatic cells.

It is clear that germ cells, somatic stem cells and probably cancer stem cells possess unique pathways for small RNA-mediated silencing. Our long-term goal is to understand how diverse RNA silencing mechanisms are integrated with other pathways in context of development and pathology. Eventually, the knowledge gained from the investigation of silencing mechanisms in stem and germ cells will help us to understand the unique biology of these cells and will impact our general understanding of gene regulation and how it is altered in disease.

Publications

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Visiting Scholars: Daniel Kahn, Jocelyn Kim
Graduate Students: Aadel Chaudhuri, Geoffrey Lovely, Arnav Mehta, Kenneth Yu, Jimmy Zhao
Undergraduates: Aarathi Minisandram, Rebecca Scholz, Nikita Sinha, Christina Weng
Staff: Joyce Chen, Yvette Garcia-Flores, Will Gibson, Christin Hong, Julie Kelly, Joanne Laurence, Claret (Siyuan) Liu, Yong Ouyang, Eric Santiestevan, Alex Tang, Yang Yu

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Summary: The Baltimore laboratory has multiple ongoing projects that all relate to aspects of immune function, cancer and infectious disease treatment and prevention. The abstracts are divided into three general topics.

The first of these relates to our efforts to engineer changes in the mouse and human immune systems using gene transfer methods. We have focused on prevention of HIV infection and on treatment of cancer. In both cases, the unmodified human immune system works poorly to attack the problems and thus, there is no effective HIV vaccine and immune therapies against cancer are at best quite transient in their effect. Thus, there is a desperate need for new approaches to which we are responding with gene-based methods.

The second of our topics is our long-standing interest in the inflammatory response, mainly in the role of the NF-κB transcription factor in this highly regulated response of the immune system. This has taken us into the study of the role microRNAs in inflammation. The study of knockout and transgenic mice for these genes has turned up a remarkable role for them as either tumor inducers or suppressors. The characterization of these roles has been an important element of our program over the last year.

The third area is a miscellaneous set of unrelated studies that represent the interests of individual trainees in the laboratory.

ENGINEERING THE IMMUNE SYSTEM TO TREAT AND PREVENT HIV INFECTION AND CANCER

148. Engineering immunity to treat cancer and HIV
Lili Yang, Yang Yu, Manorama Kalwani, Claret Siyuan Liu, David Baltimore

The concept of Engineering Immunity is to modify the immune system so it can more effectively treat particular diseases. In the past decade, we have developed methods to genetically modify various components of the immune system, including the hematopoietic stem cells (HSCs), T cells, B cells and dendritic cells (DCs) to enhance the natural immune responses against cancer and HIV. Our recent progress on these frontiers follows:

a. Engineering HSCs through TCR gene transfer to treat melanoma

We have previously developed a method to genetically program HSCs to develop into fully functional CD4 and CD8 T cells *in vivo* via retrovirus-mediated T cell receptor (TCR) gene transfer, and have proven in a mouse tumor model that this method could endow the treated mice both the ability to resist tumor challenge, and regress large, established solid tumors (Reference No. 1). In July 2005, we started collaborating with several research and clinical groups at UCLA, USC, CHLA and UCHC to move this method into clinical trials to treat melanoma. A clinical trial was launched in May 2009 and is ongoing now, having treated seven patients who generally show transient tumor reduction.

b. Engineering DCs to treat cancer and HIV

Dendritic cell (DC)-based vaccination has been widely tested in efforts to induce 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 of collecting peripheral blood from a patient, culturing DCs *in vitro*, tranducing the cells with vectors, and infusing the cells back into the same patient. Falling into the category of personal medicine, the process is costly, tedious and inconsistent. We have developed a novel method of *in situ* DC vaccination (Reference No. 2). 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 a lentivector encoding OVA. What's more, by modifying the expression pattern of OVA, we can also induce a

strong antibody response in the mouse. In the tumor challenge experiments, all mice that received such vaccination showed complete resistance to OVA tumor challenge. We have further proved in mice that such DC vaccine can induce potent T cell and antibody responses against authentic tumor antigens (such as melanoma antigens Mart-1 and Trp-2) and HIV antigens (such as Gag and Env antigens) (Reference No. 3). Currently we are evaluating the potency of this DC vaccine platform to treat cancer in a mouse B16 melanoma model and to mount anti-HIV immune responses in a non-human primate model.

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Publication

3. Dai, B., Yang, L., Yang, H., Hu, B., Baltimore, D. and Wang, P. (2009) HIV gag-specific immunity induced by a lentivector-based vaccine directed to dendritic cells. *Proc. Natl. Acad. Sci. USA* **106**(48):20382-20387.

149. Dimeric 2G12 as a potent protection against HIV-1

Xin M. Luo, Margarida Y. Y. Lei, Rana A. Feidi, Anthony P. West, Jr., Alejandro B. Balazs, Pamela J. Bjorkman, Lili Yang, David Baltimore

We previously showed *in vitro* that broadly neutralizing anti-HIV antibody 2G12 (human IgG1) could naturally form dimers that were more potent than monomeric 2G12 in neutralizing various stains of HIV-1. In this study, we have investigated *in vivo* the protective effects of monomeric versus dimeric 2G12 against HIV infection using a humanized mouse model. Our results showed that passively transferred, purified 2G12 dimer was more potent than the 2G12 monomer at preventing CD4 T cell loss and suppressing the increase of viral load following HIV infection of humanized mice. Through a backpacking approach that provides antibodies continuously, we also found that a sustained dimer concentration of 5 − 25 μg/ml or higher during the course of infection could provide effective protection against HIV. Importantly, 2G12 dimer at this concentration did not favor mutations on the HIV envelope that would cause the virus to completely escape 2G12 neutralization. We have therefore identified dimeric 2G12 as a potent HIV protective *in vivo*, which could be used as part of an antibody cocktail to prevent HIV infection.

150. Generation of mucosal immunity against HIV-1 in humanized mice through stem cell therapy

Eun Mi Hur, David Baltimore

In HIV infection, it is important to block viral transmission at mucosal sites since T cells located in mucosal area such as the gastrointestinal (GI) and genital tracts are the primary targets of initial infection in human. Immunoglobulin A (IgA) plays a critical role in mucosal immune responses by blocking viral attachment and crossing epithelial barrier where it can neutralize virus. Failures in generating antibodies that broadly neutralize primary isolates of HIV-1 suggest that alternative approaches beyond conventional immunization are needed to develop potent HIV preventive regimens. Previously, we have adopted a lentivirus vector system and humanized mouse models to explore a novel approach to generating neutralizing activity in mucosal compartments, as well as serum. We used two different humanized mouse models to verify the effect of a potent and broadly neutralizing anti-HIV antibody, human recombinant IgA2 that carries the V regions of IgG1 b12. In the Human Immune System (HIS) mouse model, human HSPCs transduced with an IgA2 b12 transgene were transplanted into $Rag2^{-/-}\gamma C^{-/-}$ mice to generate mice with a human immune system producing IgA2 b12. Another humanized mouse model, bone marrow/liver/thymus humanized mice (BLT or NOD/SCID hu-BLT mice) was used to produce b12 IgA *in vivo*. Using these animal models, we investigated the prophylactic effect of the neutralizing IgA antibodies produced in these humanized mice against HIV-1 mucosal transmission. R5 tropic HIV-1 (JR-CSF) was infected intravaginally in these BLT and HIS mice. As a result of HIV-1 mucosal challenge, marked CD4 T cell depletion was observed in intraepithelial lymphocytes (IEL) population and lamina propria lymphocytes (LPL) population of the GI tract of HIV-1 infected BLT mice. Lymphocytes isolated from female genital tract (vaginal lymphocytes, VL) also displayed a substantially reduced proportion of CD4 T cells in HIV-1 challenged BLT mice. However, BLT mice transduced with b12 IgA transgene maintained CD4 T cell population in mucosal sites even after HIV-1 mucosal challenge. This CD4 T cell depletion upon HIV-1 challenge was more obvious in mucosal sites than in peripheral blood or lymphoid organ, spleen, thymus and bone marrow. Similar results were observed in HIS humanized mouse model indicating that mucosal CD4 T cells depletion upon HIV-1 challenge was blocked or reduced by b12 IgA gene therapy. Plasma HIV-RNA was detected in HIV-challenged HIS mouse whereas no HIV-RNA was detected in the b12 IgA transduced mice group and the unchallenged group. Cells positively stained with viral protein p24 antibody were detected in immunohistochemical staining of lymphoid and mucosal tissues of the HIV challenged group of mice. The number of p24 positive cells were substantially decreased in b12 transduced humanized mice both in BLT and HIS mice models. This result suggested that b12 IgA gene transfer in a humanized mouse model confers a prophylactic effect

to HIV-1 mucosal transmission. These results support the concept that engineering human immunity using hematopoietic stem cells can reprogram the immune system *in vivo*, promoting defense against infectious agents.

151. Engineering of immunity through adeno associated vectors

Alejandro Balazs, David Baltimore

Adeno associated virus (AAV) is a small single-stranded parvovirus capable of infecting humans without causing any known illness. AAV has also been found to be capable of producing high levels of transgene expression in a variety of animal models making it an attractive gene transfer vector. We are developing AAV as a tool for the engineering of the humoral response through antibody expression from non-hematopoietic tissues such as liver and muscle. We hope to express well-characterized neutralizing antibodies against HIV using AAV to create a synthetic vaccine capable of providing long-lived protection against infection. To test this approach we are utilizing both a human immune system (HIS), as well as NOD/SCID/Gamma-HuPBMC (NSG) mouse model that can be engrafted by human hematopoietic cells to simulate a human immune system. These mice are being challenged with live HIV to determine the effectiveness of our AAV-vaccination strategy with quite significant success. This system will be used to determine the minimum quantities of antibody necessary to protect mice from challenge and identify the optimal combination of neutralizing antibodies that can provide protection.

152. Modeling the effects of variance in antigen density on bivalent antibody binding

Joshua S. Klein, David Baltimore

Antibodies are composed of two light chains and two heavy chains that combine to form two identical antigen-binding sites. Empirically, the ability to bind two physically linked antigens simultaneously (i.e., "cross-linking") can dramatically increase the apparent binding affinity of an antibody as compared to an antibody fragment with only one antigen binding site. Here we have developed a theoretical bivalent binding model that describes the dependence of the apparent affinity on the intrinsic dissociation rate constant of an antibody as a function of antigen density. Two similarly sized viruses, human immunodeficiency virus and influenza virus, that differ by more than an order of magnitude in the number of antigen spikes per virus particle, will be used to evaluate the model. A quantitative understanding of the limitations to cross-linking and the development of a general method to identify antibodies that target antigen binding sites permissible to cross-linking may advance the rational design of vaccines and immunotherapies for pathogens and other antibody targets.

153. Finding T cell receptors reactive with prostate cancer cells

Michael T. Bethune, David Baltimore

Despite its prevalence in Western men, there is no effective treatment for hormone-refractory metastatic prostate cancer. For many reasons, prostate cancer is a promising candidate for tumor-specific T cell receptor (TCR) gene therapy, an immunotherapeutic approach with demonstrated efficacy against melanoma. However, the extension of TCR gene therapy to prostate cancer will require cloned TCRs with high affinity for defined cancer-related antigens. We are using an innovative approach to obtain these TCRs and their cognate antigens. A library of peptide-MHCs (pMHCs) has been constructed from antigens over-expressed in prostate cancer. Reactive tumor-infiltrating cells isolated from clinical biopsies will be captured using this pMHC library and high-speed cell sorting. A TCR gene library will be cloned from single cells using whole genome amplification followed by PCR. The expression and functionality of cloned TCRs will be assessed using high throughput gene transfer approaches, and select candidates will be further evaluated *in vivo* using a humanized mouse model. The successful completion of this work will enable the design of TCR gene therapies and dendritic cell-targeted vaccines for prostate cancer. Moreover, the approach employed is broadly applicable to the identification of TCRs for other epithelial cancers. In parallel with these efforts, we are using computational protein design techniques to rationally engineer TCRs with enhanced characteristics for gene therapy.

STUDIES OF INFLAMMATION AND ITS RELATION TO CANCER

154. Differential control of the timing of gene expression after induction

Shengli Hao, David Baltimore

Inflammation is the host response to tissue injury and infection. It is a complicated process involving a great number of biochemical and cellular events. We are attempting to understand the molecular mechanisms that organize these inflammatory events. Particularly, we are trying to understand the mechanisms that control the temporal order of inflammatory gene activation. Using microarray gene chip analyses, as well as other techniques, we found that the temporal order of gene activation (as detected by increase of mRNA) is well conserved among different animal species, cell types and stimuli [1]. We further found that the early and late genes have different activation patterns. Messenger RNA levels are net outcome of two opposite processes: mRNA synthesis and mRNA degradation. We found mRNA stability plays a surprisingly important role in the regulation of activation pattern and mRNA stability as determined by signals in the 3'-untranslated region (UTR) of the various genes [1].

The question we are currently addressing is how the mRNA synthesis process regulates the temporal order of gene activation. In eukaryotes, mRNA synthesis includes two steps: transcription, involving synthesis of the pre-mRNA from the DNA template— including both exons and introns— and splicing, the process that removes non-coding regions (introns) and ligates protein coding regions (exons) together to make a mature mRNA. It is interesting to know whether the temporal order of gene expression is because transcription and/or splicing varies at different times or at different speeds. We have recently developed a method to quantitatively analyze the transcription and pre-mRNA splicing processes. The method is based on the quantitative measurement the pre-mRNA kinetics and pre-mRNA half lives. Using this quantitative method, we have made the following observations. 1) Compared the mature mRNA, pre-mRNAs are 5- to 200-fold less abundant. Thus, pre-mRNA kinetics is a more direct method to analyze the activity of the transcriptional machinery than mRNA accumulation kinetics. 2) Early genes are different from intermediate and late genes in transcriptional activity even in quiescent cells. Early genes are constitutively transcribed in the resting state, but intermediate and late genes have little or no transcriptional activity. 3) TNF stimulation leads to rapid transcription (i.e., within 8 minutes), earlier than that predicted from mRNA kinetics (our unpublished data). Surprisingly, transcriptional initiation of intermediate and late genes shows no or only slightly delays. This suggests that the chromatin structure modifications needed to switch from repressive state to active state can occur rapidly. 4) Transcription of early genes seems to have a uniformly rapid speed throughout the gene. In contrast, transcription of the downstream regions of intermediate and late genes displays a significant delay in time or a slow speed in contrast to their rapid initiation. This suggests control of transcriptional elongation. 5) In addition to transcription, the splicing rates of three groups of genes also show dramatic differences. These results suggest that transcriptional and post-transcriptional mechanisms coordinately determine the temporal order of inflammatory gene activation.

Publication

Hao, S. and Baltimore, D. (2009) The stability of mRNA influences the temporal order of the induction of gene activation encoding inflammatory molecules. *Nature Immunol.* **10**:281-288**.**

155. Regulation of the activity of NF-κB by methylation

Chee-Kwee Ea, David Baltimore

NF-κB is a key activator of inflammatory and immune responses with important pathological roles in cancer, heart disease and autoimmune diseases. Transcriptional activity of NF-κB is regulated by different post-translational modifications. In the course of determining if NF-κB is regulated by methylation, we identified two methyltransferases, SET9 and EHMT1, which, respectively, positively and negatively regulate the NF-κB pathway.

We discovered a novel mechanism of NF-κB regulation through lysine monomethylation by SET9 methyltransferase. Set9 specifically methylates the p65 subunit of NF-κB at lysine 37. Both TNFa and IL-1b treatments induced methylation of p65. Methylated p65 is restricted to the nucleus and this modification regulates the promoter binding of p65. Moreover, Set9-mediated methylation of p65 is required for the expression of a subset of NF-κB target genes in response to TNFa stimulation.

In contrast, EHTM1 functions as a negative regulator of both the NF-κB and the type I interferon pathways. EHMT1 catalyzes H3K9 methylation at promoters of NF-κB target gene. Moreover, EHMT1 interacts with p50 and p50 is required for repressing the expression of type I interferon-responding genes by recruiting EHMT1 to catalyze H3K9 methylation at the promoter regions. Silencing the expression of EHMT1 by RNA interference enhances expression of a subset of NF-κB regulated genes, augments interferon production and confers antiviral immunity. Our results demonstrate that EHMT1 functions as a negative regulator by catalyzing repressive H3K9 methylation at the promoter.

Publication

Ea, C.K. and Baltimore, D. (2009) Regulation of NF-kappaB activity through lysine monomethylation of p65. *Proc. Natl. Acad. Sci. USA* **106**(45):18972-7. PMCID: PMC2770010

156. Novel regulators and modulators of NF-κB activation

Parameswaran Ramakrishnan, David Baltimore

NF-κB represents a family of transcription factors playing a pivotal role in regulating the immune response, inflammation, control of apoptosis and development. We are interested in studying the regulatory events that modulate NF-κB activation and function. One aim is to determine the role of O-GlcNAc glycosylation in NF-κB action. We found that NF-κB proteins are modified by O-GlcNAcylation under hyperglycemic condition. We examined the NF-κB subunit c-Rel as a model for detailed study and identified the site of O-GlcNAcylation. By creating a non-GlcNAcylatable mutant protein, we found that this modification is essential for the NF-κB transactivation function. Currently, we are investigating the functional significance of NF-κB O-GlcNAcylation in cellular systems. While studying glycosylation of NF-κB, we also found that hyperglycemia causes enhanced survival of thymocytes in a NF-κB dependent manner both *in vitro* and *in vivo* in a mouse model. This leads to a selective survival of self-reactive thymocytes with implications in autoimmunity. We are also exploring novel mechanisms involved in NF-κB activation by Tumor Necrosis Factor and have identified a RNA binding protein as an essential component in this pathway.

157. Mechanism of Bach1-mediated transcriptional regulation and immune function

Alex So, David Baltimore

We have identified a panel of NF-κB-regulated microRNAs that regulate the immune system. Our efforts have in part been to identify common targets of these microRNAs to reveal previously unexplored signaling factors and pathways that modulate immune responses. One such target we identified is Bach1, a transcriptional regulator; importantly, little is known of the biological function of Bach1. We have carried out preliminary studies that demonstrate important roles of Bach1 in immunity. We found that Bach1 serves as a negative regulator of IFNb signaling upon stimulation with virus particles; paradoxically, it also behaves as a positive regulator upon stimulation with bacterial components. In addition, we carried out experiments *in vivo* and found that Bach1 deficient mice had defects in T helper (Th) cell functions, which modulate adoptive humoral and cell-mediated immunity. These defects correlated with an impaired ability of the deficient animals to produce antigen-specific antibodies. Importantly, the dampened Th cell response in Bach1 deficient animals also translated to a decreased disease severity in a T cell-mediated autoimmune disease model (experimental autoimmune encephalomyelitis or EAE) that mimics human multiple sclerosis. Thus, we defined a new player in immune function and have shown that Bach1 influences innate, as well as humoral and cell-mediated adoptive immunity so that its deficiency alters the incidence of immune-related disease.

158. MicroRNAs, inflammation and cancer

Ryan O'Connell, David Baltimore

Over the past year, we have been investigating the role of miRNAs in two areas of immune system biology including hematopoietic stem cells (HSCs) and autoimmune inflammation.

HSCs give rise to all blood cell lineages including cells that comprise the immune system, however, the molecular basis for HSC function remains poorly understood. We investigated whether specific miRNAs are expressed preferentially by HSCs compared to more mature bone marrow cells. Using microarray technology, we identified a subset of 11 miRNAs that are enriched in both mouse and human HSCs. To test their impacts on HSC biology, we conducted an *in vivo* gain-of-function screen that assayed the ability of miRNA-expressing HSCs to compete with control vector containing HSCs. Our results found that some miRNAs promoted hematopoietic output, while others diminished the ability of HSCs to produce downstream progeny. These observations reveal that HSC miRNAs regulate HSC homeostasis, and suggest that they function together to properly balance blood cell output by HSCs.

Of the miRNAs tested, miR-125b had the largest positive impact on engraftment. We analyzed this miRNA further, and found that graded increase in its expression caused a myeloproliferative disorder that progressed to a lethal myeloid. Thus, miR-125b is sufficient to cause cancer in the hematopoietic system when it reaches high levels of expression, suggesting a causative role in human AMLs where it has been shown to be overexpressed. We are currently working to understand how miR-125b functions at the molecular level.

In addition to our studies of early hematopoiesis, we have also continued to study the role of miR-155 in inflammation. We have set up a mouse model of human multiple sclerosis (MS), called experimental autoimmune encephalomyelitis (EAE), to study the impact of miR-155 on autoimmune inflammation. We have found that mice deficient in miR-155 are highly resistant to EAE owing largely to a defect in the development of inflammatory T lymphocytes. These findings identify a role for miR-155 in the promotion of inflammatory responses, and suggest that it may be a good therapeutic target for the treatment of certain human autoimmune disorders.

Publications

O'Connell, R.M., Chaudhuri, A.A., Rao, D.S. and Baltimore, D. (2009) Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc. Natl. Acad. Sci. USA* **106**(17):7113-8. PMCID: PMC2678424

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A. and Baltimore, D. (2010) Physiological and pathological roles for microRNAs in the immune system. *Nature Rev. Immunol.* **10**(2):111-22.

159. Role of microRNAs in B-cell development and neoplasia

Dinesh S. Rao, David Baltimore

MicroRNAs (miRNAs) can influence lineage choice or affect critical developmental checkpoints during hematopoiesis. They appear to do so by controlling the expression of critical transcription factors or other important regulators of cell fate determination. This occurs mainly via interactions of the miRNA with the 3'-untranslated region of the mRNA encoding the target protein. We became interested in the p53-induced miRNA, miR-34a in this context and have studied it intensively in B-cell development. Constitutive expression of miR-34a led to a block in B cell development at the pro-B-cell-to-pre-B-cell transition, leading to a reduction in mature B cells. This block appeared to be mediated primarily by inhibited expression of the transcription factor Foxp1. Foxp1 was a direct target of miR-34a in a 3'-untranslated region (UTR)-dependent fashion. Knockdown of Foxp1 by siRNA recapitulated the B cell developmental phenotype induced by miR-34a, whereas cotransduction of Foxp1 lacking its 3' UTR with miR-34a rescued B cell maturation. Knockdown of miR-34a resulted in increased amounts of Foxp1 and an increase in bone marrow B cells. These findings identify a role for miR-34a in connecting the p53 network with suppression of Foxp1, a known B cell oncogene.

We are also interested in the role that miR-34a plays in later B-cell development, namely the B-cell to plasma cell transition. In this context, we are studying

how p53 and miR-34a may modulate responses to T cell-dependent and T cell-independent antigens *in vitro* and following immunization *in vivo*. Lastly, we are interested in how miR-34a may modulate predicted oncogenic targets, namely BCL2, BCL6, and c-MYC, during lymphogenesis. For this last set of experiments, we are generating both murine bone marrow transfer models and developing a novel application of the humanized immune system (HIS) mouse model.

Hence our work is geared towards the understanding developmental aspects of B-cell maturation, as well as lymphomagenesis, with the goal of identifying sensitive nodes of regulation that may be controlled by microRNAs. The eventual goal is to translate this understanding of cellular biology into better treatments for patients with cancers of the hematopoietic system.

Publications

O'Connell, R.M., Rao, D.S., Chaudhuri, A.A. and Baltimore, D. (2010) Physiological and pathological roles for microRNAs in the immune system. *Nature Rev. Immunol.* **10**(2):111-22.

Rao, D.S., O'Connell, R.M., Chaudhuri, A.A., Garcia-Flores, Y., Geiger, T. and Baltimore, D. (2010) MicroRNA-34a perturbs B-cell development by targeting the Forkhead transcription factor Foxp1. *Immunity* **33**(1):48-59.

160. The role of miR-146a in maintaining hematopoietic homeostasis

Jimmy Zhao, Dinesh Rao, David Baltimore

MicroRNAs (miRNA) represent a group of evolutionarily highly conserved small noncoding RNAs that repress gene expression at the level of messenger RNA (mRNA) translation and/or stability. These small RNAs of only 19-22 nucleotide-long have been shown to play a very important role in the development and the proper functioning of the mammalian immune system. Dysregulation of global miRNA expression, as well as specific miRNA is involved in a myriad of human diseases, including autoimmune diseases and hematologic cancers.

MicroRNA-146a (miR-146a) is induced by lipopolysacharides (LPS) and several other Toll-like receptor (TLR) ligands in a NF-κB-dependent manner. And upon induction, miR-146a appears to function as a negative regulator of the NF-κB pathway by directly repressing the expression of TNF receptor-associated factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1), two of the signal transducers of the NF-κB signaling pathway. Thus, miR-146a is hypothesized to play an important role in down-regulating inflammation through a negative feedback loop. Recently, our lab has successfully generated a knockout mouse with targeted germline deletion of miR-146a. The miR-146a-/- mice developed a progressive immunoproliferative phenotype involving multiple hematopoietic lineages, massive splenomegaly, enlarged lymph nodes, and eventually malignant splenic tumors. We are currently studying the cellular lineage of the tumors and the malignant potential of the tumor, as well as trying to characterize the relationship of NF-κB signaling and miR-146a in the process of inflammation and tumorigenesis.

MISCELLANEOUS TOPICS IN INFECTIOUS DISEASE AND IMMUNOLOGY

161. Permissive secondary mutations enable influenza to develop oseltamivir resistance

Jesse Bloom, David Baltimore

The most widely used drug against influenza is oseltamivir (Tamiflu). It has long been known that H1N1 influenza strains can become resistant by acquiring a specific mutation (H274Y). However, early studies showed that this mutation severely attenuated the virus. But beginning in 2007-2008, oseltamivir-resistant seasonal H1N1 influenza began to spread rapidly through the human population. It has remained a mystery what molecular events suddenly enabled the H274Y mutation to spread.

We have used a combination of computational and experimental techniques to identify two key permissive secondary mutations. Earlier strains of seasonal H1N1 lacked these mutations and so were attenuated by H274Y. But once the two permissive mutations occurred (which appears to have happened sometime after 2000) the H274Y mutation no longer incurred a fitness penalty. This mutation therefore was rapidly able to spread.

So far, our work has both identified the permissive secondary mutations and identified the mechanisms by which they function. It appears that in earlier strains, H274Y causes a defect in the folding of the neuraminidase protein. The secondary mutations correct this defect, and thereby eliminate the deleterious effects of H274Y.

We are now working to see if this insight can be applied towards what might be termed "predictive evolutionary biology." A question of considerable medical concern is whether the new 2009 swine-origin pandemic H1N1 influenza will become resistant to oseltamivir. So far, this has only occurred in isolated cases. We have found that H274Y also causes a defect in the folding or expression of swine-origin influenza neuraminidases. We are now attempting to predict possible permissive secondary mutations that might correct this effect. We hope that this work can identify the mutations that would herald the spread of oseltamivir resistant virus.

Publications

Bloom, J.D., Gong, L.I. and Baltimore, D. (2010) Permissive secondary mutations enable the evolution of influenza oseltamivir resistance. *Science* **5983**:1272-1275.

162. Immune regulation at the maternal - fetal interface

Daniel A. Kahn, David Baltimore

The project's main objective is to better understand the maternal immune awareness of the fetus during pregnancy.

During the initial years of this project, we were able to discover that the mother indeed makes an immune response during her first pregnancy in response to specific fetal antigens. However, this immune response can be tempered by the pregnancy-induced development of a specific type of immune cell (T regulatory) that suppresses the potentially damaging anti-fetal maternal immune response.

During this year, we have attempted to understand this naturally evolved tolerance that occurs during pregnancy in response to the presence of antigenic fetuses. Specifically, we have attempted to understand better:

1. How does the mother cope with highly antigenic fetuses during a successful pregnancy?
2. What are the consequences to the fetus if the maternal tolerance response is interfered with using a drug or inflammatory stimuli?
3. What are the molecular requirements for tolerance?

In our initial system, we explored the mother's immune response against her male fetuses. Male antigen (H-Y) is a minor immunologic barrier to transplantation. We hypothesized that during a pregnancy where the fetuses each expressed major transplantation antigens, that the mother's tolerogenic profile should be augmented in order to cope with such an immunologic challenge. In order to test this hypothesis, we explored the maternal immune response to matings between C57BL/6 mothers and Balb/C fathers. These are normally successful pregnancies, despite the dramatic parental transplantation antigenic differences. We have spent a large part of this effort in isolating and producing each of the specific antigens that the fetus inherits and expressed from the father. This work is ongoing and we anticipate in the coming year to be able to fully characterize the mother's response to these specific major transplantation antigens.

To address the second question, we have probed the functional role that tolerance plays in the success of a pregnancy. For this, we used a drug used to treat recurrent cutaneous lymphoma in humans, which targets T regulatory cells for deletion. Use of this drug (ONTAK) reduced Tregs during pregnancy and we observed selective adverse outcomes for the males alone (fewer males per litter and reduced weight of the surviving males).

Recent evidence in the literature has pointed to role for c-rel—a transcription factor critical to the inflammatory response—in the development of T regulatory cells. Taking advantage of this experience, we treated mice that are deficient in c-Rel (knockout) with the drug that depletes T regulatory cells (ONTAK). In contrast with the adverse effect on the male pups when wild-type mice are treated during pregnancy, in the absence of c-Rel similarly treatment with ONTAK had is no effect on the males.

Publication

Kahn, D.A. and Baltimore, D. (2010) Pregnancy induces a fetal antigen-specific maternal T regulatory cell response that contributes to tolerance. *Proc. Nat Acad. Sci. USA* **107**:9299-9304. *Cited by Faculty of 1000 Biology 2010.

163. HIV cell-to-cell transmission decreases sensitivity to anti-retroviral therapy and may explain virus reservoir

Alex Sigal, Jocelyn T. Kim, Alejandro B. Balazs, David Baltimore

The classical description of virus infection does not take into account direct virus transmission between cells, observed for many viruses, which can potentially lead to a localized high virus levels, transmitted between the infected donor and the uninfected target cell. A well-studied example is cell-to-cell transmission in HIV infection, leading to multiple infections of some target cells.

Though direct cell-to-cell HIV transmission should not physically protect the virus from the effects of drugs used in anti-retroviral therapy which act downstream of virus entry, we showed that this mode of transmission decreases the sensitivity of infection to drugs. Infections originating from cell-free virus and cocultured infected donor cells both declined with increasing concentrations of the anti-retroviral drugs tenofovir and efavirenz. However, the rate of decline was more gradual for infections emanating from cocultured infected donor cells relative to cell-free virus. Drug concentrations that caused 100-fold drops in cell-free virus infected cells led to only 15-fold drops when HIV transmission was from cocultured infected cells. This attenuation was sufficient to keep the infection from terminating.

To explain this, we proposed a model whereby multiple infections of cells delivered by any transmission mode leads to reduced sensitivity to drugs, and validated this model using high multiplicity cell-free virus infection. Fitting the observed drug sensitivity transmission from cocultured donor cells, we obtained a mean transmission of between five to seven infectious units per target cell in the absence of drug.

The consequence of decreased sensitivity of cell-to-cell transmission to virus inhibitors may be that once some cells are infected that are capable of cell-to-cell transmission, the infection becomes more difficult to treat, potentially forming a virus reservoir.

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Undergraduate Students: Grayson Chadwick, Benjamin Steele
High School Students: Alexander Heide, Nick Pachon
Research and Laboratory Staff: Michael Anaya, Chris Foglesong, Rachel Galimidi, Priyanthi Gnanapragasam, Beth Huey Tubman, Mark Ladinsky, Lynda Llamas, Paola Marcovecchio, Marta Murphy, Caleb Ng, Pavle Nikolovsk, Maria Suzuki Politzer

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NIH
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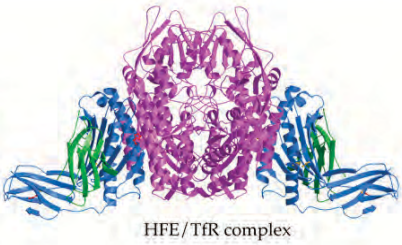
Summary: My laboratory is interested in protein-protein interactions, particularly those mediating immune recognition. We use X-ray crystallography and biochemistry to study purified proteins. Examples of crystal structures determined by our laboratory are shown in Figure 1. Some of our work focuses 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 metabolism by HFE, and serving as a chaperone for pheromone receptors in the case of M10 proteins). We are also comparing the structures and functions of host and viral Fc receptors with FcRn.

We have recently begun to include confocal and electron microcopy to examine protein complexes in cells. Some of our efforts in these areas involve the study of the trafficking pathway by which FcRn transports IgG across polarized epithelial cells, which we are examining by electron tomography and live cell confocal microscopy.

Another recent focus of our laboratory is a collaboration with David Baltimore's laboratory to "Engineer Immunity" against HIV. Our portion of the project involves designing, producing, and testing novel anti-HIV protein reagents in an effort to find proteins with increased efficacy in HIV neutralization. Promising candidates could then be administered via a gene therapy approach to HIV-infected individuals.

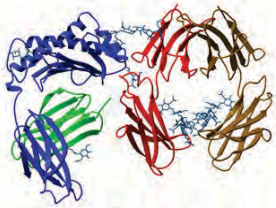
HFE



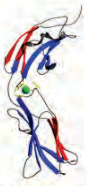
HFE/TfR complex

Hemolin

FcRn

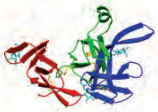


FcRn/heterodimer-Fc Complex

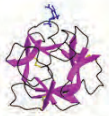


Neuroglian
Fn-III repeats

ZAG

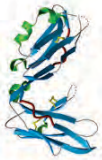


Methuselah

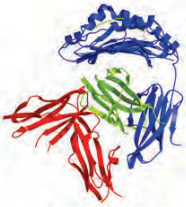


mannose receptor
cysteine-rich domain

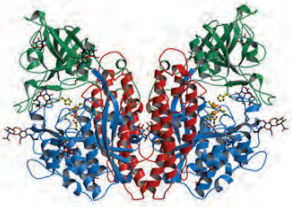
Invasin



LIR-1



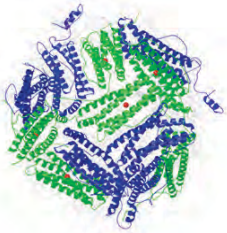
LIR-1/HLA-A2



PSMA

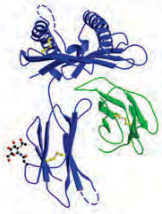
FcαRI

FcαRI/Fcα Complex

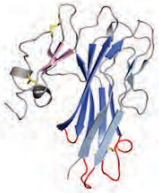


Ferritin

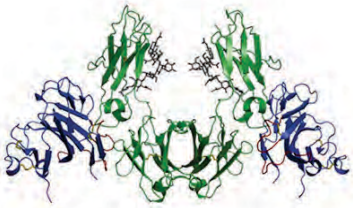
pIgR domain 1



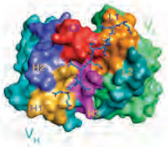
M10.5



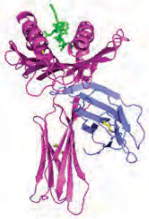
CgE



CgE/Fc Complex

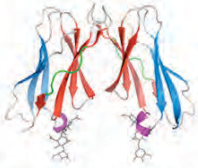


MW1 Fv/polyQ

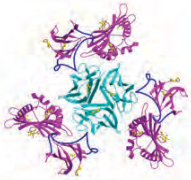


UL18

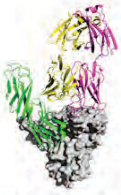
LIR-1/UL18 Complex



CHIR-AB1, an avian Fc receptor



2L/TNF Complex



HIV-1 clade C gp120/Fab/CD4

Neogenin FnIII5-6

164. Electron tomographic studies of FcRn-mediated antibody transport across epithelial cells

Mark S. Ladinsky, Kathryn E. Huey-Tubman

The neonatal Fc receptor (FcRn) transports maternal IgG across epithelial barriers, thereby providing the fetus or newborn with humoral immunity before its immune system is fully functional. In newborn rodents, FcRn transfers IgG from milk to blood by apical-to-basolateral transcytosis across intestinal epithelial cells. As milk passes through the neonatal digestive system, maternal IgG is removed by FcRn-expressing cells in the proximal small intestine (duodenum and jejunum). Remaining proteins are absorbed and degraded by FcRn-negative cells in the distal small intestine (ileum). We are using a method developed in our lab (1) to enhance and detect individual nanogold-labeled Fc within intestinal epithelial cells and are using this method, in conjunction with electron tomography and immuno-electron microscopy, to directly visualize the transcytotic pathway in jejunal cells (2). We are using multi-frame montaging of serial sections to generate large-area tomographic reconstructions in order to draw a more complete picture of FcRn-mediated transcytosis. We are following gold-labeled Fc in the basolateral portions of jejunal cells to characterize the compartments responsible for the exit of IgG from the intestinal epithelium to the bloodstream. We are also comparing jejunal structures with their counterparts in duodenum and contrasting them with FcRn-positive fluid-phase endocytic compartments in the ileum. Finally we are comparing our results with equivalent data from older (weaned) animals, in which FcRn-mediated Fc transport has stopped, to characterize structural changes to the endocytic pathway as the animal matures and loses dependence on maternal IgG. Additional studies are focusing on FcRn expression and localization in immortalized cell lines (MDCK and IEC-6); model systems that can be experimentally perturbed in ways that are not practical in animal systems. Our results to date show that Fc moves through complex networks of entangled tubules and irregular vesicles during transcytosis and may pool in dilated domains of the lateral intercellular space during or following exit from the basolateral side of the cell. We have elucidated more than 30 new structural features of transcytosis and demonstrated a dramatic spatial complexity of the process, at both the structural and molecular levels. New studies of transcytosis, using these approaches in other systems, will further expand our understanding of FcRn's role in other intracellular trafficking systems.

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2. He, W., Ladinsky, M.S., Huey-Tubman K.E., Jensen, G.J., McIntosh, J.R. and Bjorkman, P.J. (2008) *Nature* **455**:542-546.

165. Comparison of FcRn- and pIgR-mediated transport in MDCK cells by fluorescence confocal microscopy

Galina V. Jerdeva, Devin B. Tesar[1], Kathryn E. Huey-Tubman, Mark S. Ladinsky, Scott E. Fraser[2]

Protein delivery across polarized epithelia is controlled by receptor-mediated transcytosis. Many studies have examined basolateral-to-apical trafficking of polymeric IgA (pIgA) by the polymeric immunoglobulin receptor (pIgR). Less is known about apical-to-basolateral transcytosis, the direction the neonatal Fc receptor (FcRn) transports maternal IgGs across intestinal epithelia. To compare apical-to-basolateral and basolateral-to-apical transcytosis, we co-expressed FcRn and pIgR in Madin-Darby canine kidney (MDCK) cells and used pulse-chase experiments with confocal microscopy to examine transport of apically applied IgG Fcγ and basolaterally applied pIgA. Fcγ and pIgA trafficking routes were initially separate but intermixed at later chase times. Fcγ was first localized near the apical surface, but became more equally distributed across the cell, consistent with concomitant transcytosis and recycling. By contrast, pIgA transport was strongly unidirectional: pIgA shifted from near the basolateral surface to an apical location with increasing time. Some Fcγ and pIgA fluorescence colocalized in early (EEA1-positive), recycling (Rab11a-positive), and transferring (Tf)-positive common/basolateral recycling endosomes. Fcγ became more enriched in Tf-positive endosomes with time, whereas pIgA was sorted from these compartments. Live-cell imaging revealed that vesicles containing Fcγ or pIgA shared similar mobility characteristics and were equivalently affected by depolymerizing microtubules, indicating that both trafficking routes depended to roughly the same extent on intact microtubules.

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[2]*Anna L. Rosen Professor of Biology and Professor of Bioengineering; Director, Donna and Benjamin M. Rosen Bioengineering Center, Caltech*

Publication

Jerdeva, G.V., Tesar, D.B., Huey-Tubman, K.E., Ladinsky, M.S., Fraser, S.E. and Bjorkman, P.J. (2010) Comparison of FcRn- and pIgR-mediated transport in MDCK cells by fluorescence confocal microscopy. *Traffic* **11**:1205-1220.

166. Nanogold as a specific marker for electron cryotomography

*Yongning He, Grant J. Jensen**

While electron cryotomography provides "molecular" resolution, three-dimensional images of unique biological specimens, sample crowdedness, and/or resolution limitations can make it difficult to identify specific macromolecular components. Here we used a 1.4 nm Nanogold® cluster specifically attached to the Fc fragment of IgG to monitor its interaction with the neonatal Fc receptor (FcRn), a membrane-bound receptor that transports IgG across cells in acidic intracellular vesicles. ECT was used to image complexes formed by

Nanogold-labeled Fc bound to FcRn attached to the outer surface of synthetic liposomes. In the resulting three-dimensional reconstructions, 1.4 nm Nanogold particles were distributed predominantly along the interfaces where 2:1 FcRn-Fc complexes bridged adjacent lipid bilayers. These results demonstrate that the 1.4 nm Nanogold cluster is visible in tomograms of typically thick samples ~(250 nm) recorded with defocuses appropriate for large macromolecules and is thus, an effective marker.

[*]*Associate Professor, Division of Biology, Caltech*

Reference

He, Y., Jensen, G.J. and Bjorkman, P.J. (2009) Nanogold as a specific marker for electron cryotomography. *Microscopy and Microanal*. **15**:183-188. Selected as the best Biological Sciences paper in *Microscopy and Microanalysis*.

167. Cryo-electron tomography of homophilic adhesion mediated by the neural cell adhesion molecule L1

Yongning He, Grant J. Jensen[*] Pamela J. Bjorkman

The neural cell adhesion molecule L1 participates in homophilic interactions important for axon guidance and neuronal development. The structural details of homophilic adhesion mediated by L1 and other immunoglobulin superfamily members containing an N-terminal horseshoe arrangement of four immunoglobulin-like domains are unknown. Here we used cryo-electron tomography to study liposomes to which intact or truncated forms of the L1 ectodomain were attached. Tomographic reconstructions revealed an adhesion interface with a regular and repeating pattern consistent with interactions between paired horseshoes contributed by L1 proteins from neighboring liposomes. The characteristics of the pattern changed when N-linked carbohydrates were altered by removing sialic acids or converting from complex to high mannose or oligomannose glycans, suggesting a regulatory role for carbohydrates in L1-mediated homophilic adhesion. Using the results from tomograms and crystal structures of L1-related molecules, we present a structural model for L1-mediated homophilic adhesion that depends on protein-protein, protein-carbohydrate, and carbohydrate-carbohydrate interactions.

[*]*Associate Professor, Division of Biology, Caltech*

Publication

He, Y., Jensen, G.J. and Bjorkman, P.J. (2009) Cryoelectron tomography of homophilic adhesion mediated by the neural cell adhesion molecule L1. *Structure* **17**: 460-471.

168. Biophysical approach to understanding L1-mediated homophilic adhesion

Fan Yang, Tristan Ursell[1], Rob Phillips[2]

L1 is a transmembrane cell adhesion molecule. L1-mediated homophilic and heterophilic adhesion events are critical in neural cell recognition. Current models for homophilic adhesion by L1 include a domain-swapping model, the zipper model, and a carbohydrate-dependent model proposed based on cryo-electron tomography study by Yongning He from our lab (ref. 1). In this study, we aim to investigate L1-mediated homophilic adhesion using a combination of computational and experimental tools. We developed a confocal microscopy assay using giant unilamellar vesicles (GUV) as an experimental platform. Recombinant L1 with 6x-His-tag was reconstituted in these GUVs and coated on copper-NTA functionalized glass slides, as well. L1-coated GUVs are added on top of a glass slide and confocal images are taken to reconstruct the three-dimensional shape of the GUVs adhering to glass surface. We proposed a three-parameter basic shape model based on membrane mechanics to describe an adhering vesicle and evaluate adhesion energy on the basis of vesicle configuration. Simulations with Matlab revealed that under a low to moderate adhesion strength regime, adhesion strength can be derived by fitting experimental data to our model. However, under a high adhesion strength regime, the shape change is probably too small to resolve by confocal microscopy. Confocal data sets have been analyzed using our model and adhesion strengths were derived.

[1]*Postdoctoral Fellow, Stanford University*
[2]*Professor of Applied Physics and Mechanical Engineering, Caltech*

169. Structural studies of HIV Env proteins from Clade C viruses

Ron Diskin, Paola Marcovecchio

Since the appearance of HIV/AIDS more than two decades ago, over 20 million people worldwide have died from AIDS and about twice as many are currently infected with various strains of HIV-1. Viruses classified as clade C are the most common type of HIV that is rapidly spreading in the world. In order to combat HIV and develop an efficient vaccine, we need structural information of the functional envelope proteins that allow penetration of the virus into the host cell. This structural knowledge needs to encompass many diverse HIV-1 strains. We recently presented the first crystal structure of a clade C gp120 spike protein. The structure is of a complex between gp120 from the CAP210.2.00.E8 strain, the host receptor CD4 and the CD4-induced antibody 21c. The crystal structure reveals that the 21c epitope involves contacts with the viral gp120, a nonself antigen, and with the T-cell receptor CD4, an autoantigen. Binding studies in solution using wild-type and mutant CD4 show that 21c Fab is binding CD4 in the absence of gp120, and that binding of 21c to other gp120s from different HIV strains requires the crystallographically observed 21c-CD4 interaction. This crystal structure facilitates a cross clade structural analysis and provides the first visualization of an

autoreactive antibody Fab complexed with both the self and the nonself antigens.

Publication

Diskin, R., Marcovecchio, P.M. and Bjorkman, P.J. (2010) Structure of a clade C HIV gp120 plus CD4 and a CD4-induced antibody reveals anti-CD4 polyreactivity. *Nature Struct. Mol. Biol.* **17**:608-613.

170. Redesigning antibodies: overcoming limitations to antibody-mediated neutralization of HIV

Joshua S. Klein, Rachel P. Galimidi, Priyanthi N. P. Gnanapragasam, Chris P. Foglesong, Maria Suzuki, Anthony P. West Jr.

Researchers have been focused for more than 20 years on explaining why HIV so effectively evades neutralization by antibodies. Electron micrographs of HIV have shown that each virus particle has on average fewer than 15 envelope spikes. A nearest neighbor distance analysis showed that most of the spikes are separated by distances greater than the approximately 10 to 15 nm reach of the two Fabs of an IgG, as are the distances between repeating epitopes on a single spike trimer, suggesting that IgGs are generally unable to bind bivalently to HIV. In an attempt to improve the neutralization potencies of monoclonal antibodies to HIV, we are developing protein fusion extensions designed to adopt extended conformations for insertion into the hinge regions separating the Fabs from the Fc in an IgG. These redesigned antibodies may be used as passive immunotherapies and gene therapies to treat and prevent HIV infection, respectively.

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171. Evaluation of CD4-CD4i antibody architectures yields potent, broadly cross-reactive anti-HIV reagents

Anthony P. West, Jr., Rachel P. Galimidi, Christopher P. Foglesong, Priyanthi N.P. Gnanapragasam, Joshua S. Klein

The envelope glycoprotein of Human Immunodeficiency Virus-1 (HIV-1) has several adaptations that allow the virus to evade antibody neutralization. Nevertheless, a few broadly cross reactive neutralizing antibodies, as well as reagents containing portions of CD4, the HIV receptor, have demonstrated partial efficacy in suppressing viral replication. One type of reagent designed for improved HIV neutralization fuses the CD4 D1-D2 domains to the variable regions of an antibody recognizing the CD4-induced (CD4i) coreceptor-binding site on the gp120 portion of the HIV envelope spike. We designed, expressed, purified, and tested the neutralization potencies of CD4-CD4i antibody reagents with different architectures, antibody combining sites, and linkers. We found that fusing CD4 to the heavy-chain of the CD4i antibody E51 yields a bivalent reagent including an antibody Fc region that expresses well, is expected to have a long serum half-life, and has comparable to or greater neutralization activity than well-known broadly neutralizing anti-HIV antibodies. A CD4 fusion with the anti-HIV carbohydrate antibody 2G12 also results in a potent neutralizing reagent with more broadly neutralizing activity than 2G12 alone.

Publication

West, A.P., Jr., Galimidi, R.P., Foglesong, C.P., Gnanapragasam, P.N.P., Klein, J.S. and Bjorkman, P.J. (2010) Evaluation of alternative CD4-CD4i antibody architectures yields potent, broadly cross-reactive anti-HIV reagents. *J. Virol.* **84**:261-269.

172. Structural studies of 2G12, a broadly neutralizing antibody against HIV-1

Yunji Wu, Matt Thornton

The HIV-1 envelope spike protein is one of the most heavily glycosylated in nature, with 50% of its molecular weight attributable to surface carbohydrates. The host-derived thick glycan shield on the envelope spike is one of the main barriers to generating potent, broadly neutralizing antibodies against HIV-1. 2G12 is a broadly neutralizing monoclonal antibody that binds to a high-mannose epitope on the gp120 subunit of the HIV-1 envelope spike. 2G12 features a unique 3D structure in which the two Fab arms exchange variable heavy domains, creating two additional unique combining sites. In addition, our group recently discovered a dimeric form of 2G12 which exhibits a 50- to 80-fold increase in neutralization potency over the monomeric form when assessed over several Clade A and B strains of HIV-1. Current efforts are aimed at: (1) determining the structure of dimeric 2G12; (2) elucidating the mechanism of increased potency of the 2G12 dimer over the monomer, and, (3) characterizing the exact nature of the interaction between gp120 and both forms of 2G12. To accomplish this, we will employ x-ray crystallography, as well as biophysical assays such as Surface Plasmon Resonance. Knowledge about the roles of 2G12 domain-exchange and dimerization in binding and neutralizing activity will provide valuable insight for the design and discovery of more effective anti-HIV therapeutic agents, as well as the elusive HIV vaccine.

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Professor of Biology, 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

173. Dynein-driven oscillation resulting from doublet separation and reassociation

Charles J. Brokaw

When ATP is supplied to partially disintegrated, demembranated flagella, where the outer doublets remain attached at the basal end, sliding of one doublet towards the basal end of an adjacent, substrate doublet causes it to bulge out and separate away from the adjacent doublet. Further sliding in the distal region is accommodated by enlargement of the separation and finally by complete separation. This followed by reassociation of the separated doublet, starting from the basal end where it is held close to its partner. This sequence is repeated at a regular frequency. This situation, and other examples where doublets separate and reassociate, suggest that dyneins are not only the flagellar motors, but are also responsible for maintaining the proper operating distance between doublets. Although obviously this is a pathological situation, it is the only example of dynein-driven oscillation that can be explained by obvious on and off states, resulting from proximity to or separation from, the partner doublet.

Computer simulation of this behavior is being used to gain a better understanding of dynein function. Although based on previous simulations of flagellar and microtubular bending, it incorporates the new feature of a variable distance between two filaments. Last year's work confirmed the basic explanation of the oscillation by separation-dependent on and off states of dynein activity, and demonstrated the need for dyneins to provide an adhesive force as well as a sliding force.

Programming has now been extended to remove a requirement for stabilization by providing a higher bending resistance for the substrate doublet. With both doublets allowed to bend, the system still gives movements similar (albeit not precisely identical) to the experimental results. The programming has also been extended to incorporate a row of 933 individual dyneins, each behaving stochastically, so that detailed models for dynein mechanochemical cycling can be evaluated. Realistic results are obtained with a dynein model in which the sliding force and adhesive force are orthogonal, and independent except for the dependence of attachment-detachment reactions on the sum of the adhesive and sliding elastic energies. Unrealistic results are obtained with an alternative model, in which both the adhesive force and the sliding force result from one simple elastic link between a dynein on the bending doublet and a binding site on the substrate doublet. The former model appears to be more consistent with current structural information about dyneins *in situ*.

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Postdoctoral Scholars: Kenny Karanja, Suhash Pokharel
Member of the Professional Staff: Martin E. Budd, Piotr Polaczek
Lecturer: Betty Bertani
Graduate Student: Barbara Kraatz-Fortini
Research and Laboratory Staff: Stephanie Cox, 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 and treatment of cancer. Our lab studies the components of the DNA replication apparatus that promote genomic stability. We use yeast genetics and biochemistry, *Xenopus* egg extracts, and human cells.

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 a few 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 polymerases, helicases, and nucleases required for this processing constitute an ongoing mechanistic biochemistry project in the laboratory. Okazaki fragment processing represents the heart of the replication machine, and our studies have revealed that, as in prokaryotes, the replisome is not a machine made up of dedicated parts like its namesake the ribosome. Instead, the replisome is a dynamic structure with proteins constantly exchanging protein and DNA partners to coordinate the rapid and high fidelity synthesis of the anti-parallel leading and lagging strands of the DNA template. Our current work focuses on the regulation, by reversible acetylation and phosphorylation, of the protein/protein and protein/DNA hand-offs that we have defined over the last decade.

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 that interact with Dna2. Martin Budd and Laura Hoopes found that *dna2* mutants have a significantly reduced life span. Microarray analysis by Isabelle Lesur showed that the *dna2* mutants age by the same pathway as wildtype cells; they just age faster. Interestingly, the human Bloom and Werner genes complement the replication defect of *dna2* mutants, suggesting that Dna2 works in the same pathway with these genes. We have now shown that the Dna2 helicase works with the yeast BLM ortholog, Sgs1, in the major pathway of double-strand break repair in yeast and are studying the same process in both yeast and human cells. Together Dna2 and Sgs1 are involved in the initial resection of the 5' terminated strand of the DSB to produce a single-stranded 3' end. This is a crucial step because it is where the cell decides whether to pursue the relatively error-free homologous recombination pathway or the more error-prone non-homologous end joining repair. The 3' end generated by Dna2/Sgs1 is involved in strand invasion of the homolog and thus, the initiation of strand exchange. Perhaps even more important the single-stranded DNA is a key intermediate in the activation of the cell cycle checkpoint that protects the cell from genome instability in the presence of a double-strand break arising from replication fork failure. In collaboration with Dunphy lab, we readily showed that Dna2 also participates in resection in *Xenopus* egg extracts. We have now reconstituted the recombination machine both from purified yeast proteins and from purified human counterparts, including Dna2 and BLM helicase. BLM helicase is defective in one of the most cancer-prone diseases yet described, Bloom syndrome. Cells from these patients show a high frequency of sister chromatid exchanges and quadriradials. The biochemical approach provides a mechanistic basis for this dynamic recombination processing machine. Especially for the human proteins, this provides insights previously unavailable due to the difficulty of performing recombination experiments in human cells.

Telomeres, i.e., the ends of linear chromosomes, are a special case of the type of ends found at DSBs. Not surprisingly, Dna2 also plays a significant role at telomeres. In fact, the bulk of Dna2 is localized to telomeres and, in yeast, this localization is dynamic. During G1 and G2 phases of the cell cycle, Dna2 is at telomeres. During S phase Dna2 leaves telomeres and is present on the replicating chromatin. Dna2 is also mobilized from telomeres in response to the induction of intrachromosomal double-strand breaks with agents such as bleomycin. At the end of S phase, telomeres become single-stranded in all organisms and this occurs through 5' resection to produce single-stranded 3' overhangs. We have now shown that Dna2 is one of the major enzymes involved in resection at telomeres, as well as internal DSBs. It will be important to investigate if the same holds true in human cells with Dna2 knocked down by shRNA.

174. Customizing the shape of magnetite crystals

Andrew Freddo[1], Ali Hajimiri[2], Cody Nash[3], Elizabeth Bertani

Magnetotactic bacteria, such as *Magnetospirillum magnetotacticum*, synthesize crystals of the magnetic iron ore, magnetite, by pumping environmental iron into preformed organelles called magnetosomes. Different species of magnetotactic bacteria synthesize magnetite crystals of different shapes, which can be distinguished by microscopy. Knowledge of the factors that affect crystal shape would aid in the production of customized magnetite crystals for specialized purposes.

The C-terminal ends of four different *Magnetospirillum* proteins have been found tightly bound to magnetite crystals and the genes producing these proteins identified. The C-terminal end of one such protein has been reported (Arakaki *et al*., 2003) to influence the shape of magnetite crystals when it is added during their formation in an *in vitro* system. In order to broaden the importance of this observation, we have begun a project to clone similar genes from other magnetotactic organisms, overexpress the C-terminal fragments of the proteins, and test them singly or in groups for their possible effect on shape during crystal formation in an *in vitro* system.

Last year, we cloned into an expression vector, the relevant genes from *Magnetospirillum* strain AMB-1 and from Magnetococcus, strain MC-1. These bacteria produce cubo-octahedral or elongate hexagonal crystals, respectfully. This year the project moved on to purifying the C-terminal fragments of the proteins and testing them in an *in vitro* system for their possible effect on shape during crystal formation. Some technical difficulties, resulting from the small size of the fragments (6 and 13 kD) and inefficient removal of the tags from the fusion proteins by protease, still need to be resolved, but it was confirmed that magnetite crystals are being produced by the *in vitro* system.

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175. Regulation of telomere structure and length by DNA2, PIF1, MRE11 and EXO1

Martin Budd, Judith Campbell

The appearance of a 3' single-stranded tails at a chromosome end is a critical step in the maintenance of telomeres. A single-stranded 3' GT tail is required to target Telomerase to chromosome ends and to hybridize to the RNA primer of the enzyme. Identifying the nucleases or helicases involved in creating the 3' tail is critical in understanding of the first step in telomere synthesis. One nuclease involved is Mre11 since 3' GT tails are shorter in mre11Δ mutants. Dna2 is a 5' to 3' helicase, a exo/endonuclease that processes Okazaki fragments with Rad27. Repair of Double-strand Breaks (DSB) is blocked in the mutants deleted for Dna2 and mutated in the Mre11 nuclease. The question was addressed with two techniques, a *de-novo* telomere synthesis assay, and another assay measuring single-stranded DNA at endogenous Y' telomeres. *dna2-1* strains were defective in telomere elongation at a short telomere created at an endonuclease induced double-stand break. A defect in exonuclease resection was observed at the GT/CA telomere end of the DSB in the *dna2-1* strain. To test for interactions of additional nucleases with dna2 in telomere resection the appearance of single-stranded DNA in strains was assayed in *pif1Δ*, *dna2*Δ *pif1*Δ, and *mre11*(nuclease minus) *dna2*Δ *pif1*Δ strains. Single-stranded 3' GT overhangs appeared in mutants deleted in dna2 and with an inactive mre11 nuclease. Either another nuclease besides Dna2 or Mre11 can process telomere ends to 3' overhangs or long overhangs can arise as a result of Okazaki Fragment synthesis in the *dna2*Δ *pif1*Δ *mre11-H125N* strain. We have also examined Okazaki fragment synthesis in strains defective for Dna2 and Exo1 by assaying for S phase single-stranded DNA and have found the *dna2-2 exo1Δ* mutant is defective. Telomere length experiments were done to elucidate the interactions of Pif1, Dna2, Mre11, and Tel1.

176. DNA end resection by Dna2-Sgs1-RPA and its stimulation by Top3-Rmi1 and Mre11-Rad50-Xrs2

Petr Cejka[1,2], Elda Cannavo[1,2], Piotr Polaczek, Taro Masuda-Sasa, Subhash Pokharel, Judith L. Campbell, Stephen C. Kowalczykowski[1,2]

The repair of DNA double-strand breaks (DSBs) by homologous recombination (HR) requires processing of broken ends. For repair to commence, the DSB must first be resected to generate a 3'-single-stranded DNA (ssDNA) overhang, which becomes a substrate for the DNA strand exchange protein, Rad51. Genetic studies have implicated a multitude of proteins in the process, including helicases, nucleases, and topoisomerases. Here we have biochemically reconstituted elements of the resection process and reveal that it requires the nuclease, Dna2, the RecQ-family helicase, Sgs1, and the ssDNA-binding protein, Replication protein-A (RPA). We establish that Dna2, Sgs1, and RPA comprise a minimal protein complex capable of DNA resection *in vitro*. Sgs1 helicase unwinds the DNA to produce an intermediate that is digested by Dna2, and RPA stimulates DNA unwinding by Sgs1 in a species-specific manner. Interestingly, RPA is also required both to direct Dna2 nucleolytic activity to the 5'-terminated strand of the DNA break and to inhibit 3'→5' degradation by Dna2, actions which generate and protect the 3'-ssDNA overhang, respectively. In addition to this core machinery, we establish that both the topoisomerase 3 (Top3) and Rmi1 complex and the Mre11-Rad50-Xrs2 complex (MRX) play important roles as stimulatory components. Stimulation of end resection by the Top3-Rmi1 heterodimer and the MRX proteins is *via* complex formation with Sgs1[6] that unexpectedly stimulates DNA

unwinding. We suggest that Top3-Rmi1 and MRX are important for recruitment of the Sgs1-Dna2 complex to DSBs. Our experiments confirm recent seminal genetic observations, and provide a mechanistic framework for understanding initial steps of recombinational DNA repair in eukaryotes.

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177. Investigating the role of human Dna2 nuclease/helicase in DNA replication and recombinational repair

Kenneth Karanja, Stephanie Cox

Dna2, an essential helicase/nuclease in yeast and *Xenopus*, is an important player in the maintenance of genome integrity during DNA replication and repair. Dna2 is involved in lagging-strand replication where it is required for removal of the RNA-DNA primers that initiate the synthesis of Okazaki fragments. In addition, during double-stranded break (DSB) repair, Dna2 has recently been shown to act in concert with the MRN/X (Mre11, Rad50, Nbs1 in humans and Xrs1 in yeast) complex to process the DSB into substrates suitable for homologous recombination.

Our research seeks to elucidate the role of human Dna2 in the replication and repair process. During DNA replication, we have found that Dna2-depleted human cells are sensitive to methyl methane sulfonate and hydroxyurea. Cell viability significantly decreases with MMS treatment whereas HU treatment causes S-phase arrest. Moreover, we have evidence that Dna2 interacts with Ctf4/And-1 and MCM10, two members of the multi-protein replisome progression complex found at nascent DNA replication forks. Taken together, we postulate that Dna2 is important for proper cell cycle progression.

DNA interstrand cross-links (ICL) are lesions caused by cisplatin and mitomycin C that are repaired by recombinational repair of the Fanconi anemia pathway. To understand the role of Dna2 in ICL, we tested the sensitivity of Dna2-depleted cells to cisplatin and mitomycin C. Cells exhibit mild sensitivity to both drugs, perhaps due to the presence of other compensatory nucleases such as Exo1. However, when Dna2 was depleted in cells already deficient for FancD2, a key protein in ICL repair, cells were more tolerant to DNA damage. We have also found that Dna2 interacts physically in cells with FancD2. This interaction warrants further investigation since FancD2 is suggested to have a role in both DNA replication initiation and repair of stalled replication forks. These results provide an impetus for further investigation of the role of Dna2 in replication and repair.

178. Post-translational modification of Dna2 following DNA damage

Barbara Kraatz-Fortini, J.L. Campbell

Saccharomyces cerevisiae Dna2 is an essential enzyme with helicase, nuclease, ATPase, ssDNA annealing and strand exchange enzymatic activities. In addition to an established role in Okazaki fragment processing and telomere maintenance, both genetic and biochemical evidence implicates Dna2 in the repair of double-strand breaks and other forms of DNA damage. Both helicase- and nuclease-defective mutants of Dna2 are known to be sensitive to DNA damaging agents. Further, the Dna2 nuclease, together with the MRX complex and Sgs1, is important for the efficient resection of double-strand breaks prior to homologous recombination.

We find that the modulation of Dna2 activity after DNA damage depends on the signaling of multiple kinases. Prominent among these is the DNA damage sensor kinase Mec1, which phosphorylates Dna2 after multiple forms of DNA damage. Post-translational modification of Dna2 is important for DNA damage survival as several non-phosphorylatable point-mutants of Dna2 show dramatic sensitivity to treatment with the DNA damaging agent MMS and replication fork stalling agent hydroxyurea. Two of these phosphorylation sites reside in the N-terminal third of the large Dna2 protein. This poorly characterized region is distinct from the nuclease and helicase enzymatic domains and is dispensable for growth at 30 degrees. Further studies must address how this "regulatory" domain modulates the function of the Dna2 enzyme following DNA damage induced phosphorylation.

179. BLM-DNA2-RPA-MRN and EXO1-BLM-RPA-MRN constitute two DNA end resection machineries for human DNA-break repair

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Repair of double-strand DNA-breaks requires processing to produce 3'-terminated single-stranded DNA. We have biochemically reconstituted DNA end resection using purified human proteins: Bloom helicase (BLM), DNA2 helicase/nuclease, Exonuclease 1 (EXO1), the complex comprising MRE11, RAD50, and NBS1 (MRN), and Replication protein-A (RPA). Resection occurs *via* two routes. In one, BLM and DNA2 physically and specifically interact to resect DNA in a process that is ATP-dependent, and requires BLM helicase and DNA2 nuclease functions. RPA is essential for both DNA unwinding by BLM and enforcing 5'→3' resection polarity by DNA2. MRN accelerates processing by recruiting BLM to the end. In the other, EXO1 resects the DNA, and is stimulated by BLM, MRN, and RPA. BLM increases the affinity of EXO1 for ends, and MRN recruits and enhances the processivity of EXO1. Our results establish two of the core machineries that initiate recombinational DNA repair in human cells.

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180. Structure/activity analysis of Dna2 mutants and the role of acetylation in Dna2

Subhash Pokharel

Although it is agreed that the binding of Dna2 with DNA substrates is structure-specific, there is no consensus among researchers in the field about the mode of binding of either the nuclease or helicase domains of Dna2 to DNA substrates in Okazaki fragment processing (OFP), long patch base excision repair (LP-BER) or double-strand break repair. To answer those questions, further biochemical structure/function analysis of Dna2 needs to be carried out. For the purpose, we have cloned yeast Dna2 gene in a new yeast expression vector, which gives 17 mg of protein per liter of culture, sufficient to begin crystallization trials. Sequence alignments demonstrate that the Dna2 helicase domain exhibits the conserved motifs of Super Family I (SFI) DNA and RNA helicases. Motifs Ia, III, and V are implicated in DNA binding by previous studies of other SFI helicases. The residues in these conserved motifs will be mutated and characterized using oligonucleotides mimicking known substrates of Dna2 in OFP and LP-BER.

Recently, we have reported that human Dna2 is acetylated by the KAT/transcriptional coactivator protein, p300, *in vitro*. The acetylation of Dna2 stimulates both 5'-3' endonuclease and binding activities over 12-fold. Similarly, we found that the RTT109/Vps75 acetylase complex, a functional and structural counterpart to p300, activates the nuclease activity of yeast Dna2. Furthermore, the yeast Dna2 expressed in yeast was found to be acetylated indicating a cellular role of this post-translational modification. Moreover, human Dna2 was also found to be acetylated *in vivo*, and the degree of acetylation increased after UV irradiation, further supporting the possible cellular role of this modification. In collaboration with John Yates, Scripps Institute, we have mapped the lysines acetylated by p300 and RTT109, respectively. We have created non-acetylatable K to R mutants using site-directed mutagenesis. These mutant proteins will be characterized by both *in vitro* assay and *in vivo* complementation studies to determine the role of acetylation in the regulation of Dna2 in DNA replication and homologous recombinational repair.

181. Biochemical characterization of the multifunctional Dna2 helicase/nuclease

Piotr Polaczek, Lu Chen, Subhash Pokharel

The main focus of our research in the past year was further biochemical characterization of the multifunctional protein Dna2. To that end, we have accomplished the following:

1. We have shown that *Xenopus* Dna2 possesses helicase activity and thus, is a conserved feature of the protein from yeast to man. This activity was revealed with the use of a nuclease-dead variant of the protein. In wt Dna2 the strong nuclease activity of the protein prevented detection of the helicase.
2. We have previously shown that RPA single-stranded DNA binding protein stimulates 5'–end cleavage of DNA substrates by Dna2 and inhibits 3'- end cleavage. Now, we and others have shown that this feature of Dna2 is critical for the strand invasion step of homologous recombination and consequently the preservation of genomic stability, where it collaborates with the Sgs1 helicase to form the previously unrecognized eukaryotic equivalent of the prokaryotic/Archaeal resection machine RecBCD/AddAB.
3. We have further characterized the biochemical properties of the yeast Dna2 helicase. We have found that ATP/Mg^{2+} ratio of 2:1, previously shown to be inhibitory to Dna2 exonuclease, completely blocks the endonuclease activity of the protein on circular molecules. Without the endonucleolytic activity Dna2 cannot unwind fully annealed oligonucleotides to circular molecules. This provides evidence that, unlike other known SF1 family helicases, Dna2 requires a 5' single-stranded end in this process.

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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 not only the overall morphology of mitochondria in cells, but also 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?
(4) How are mitochondrial genomes packaged and maintained?
(5) What regulatory mechanisms maintain the quality of mitochondria?
To address these issues, we use a wide range of approaches, including genetics, biochemistry, cell biology, and structural biology.

I. Cellular and physiological functions of mitochondrial fusion and fission

A typical mammalian cell can have hundreds of mitochondria. However, each mitochondrion is not an autonomous organelle, 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.

Much of our work focuses on proteins involved in mitochondrial fusion or fission. Three large GTPases are essential for mitochondrial fusion. The mitofusins (Mfn1 and Mfn2) are transmembrane GTPases embedded in the outer membrane of mitochondria, and OPA1 is a dynamin-related protein localized to the intermembrane space. Our analysis of cells lacking mitofusins or OPA1 indicates that these proteins act at distinct steps during the membrane fusion process. Mitochondrial fission requires the function of Drp1, a dynamin-related protein that recruited to the mitochondrial surface to promote fission.

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, indicating an essential function for mitofusins during embryogenesis. In both cases, the embryonic lethality results from placental dysfunction. From mouse models, we have generated cellular systems to dissect mitochondrial dynamics. Embryonic fibroblasts lacking Mfn1 or Mfn2 display fragmented mitochondria, a phenotype due to a severe reduction in mitochondrial fusion. Cells lacking OPA1 or 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. In part, these defects arise from a loss of mtDNA nucleoids, suggesting that content mixing due to mitochondrial fusion plays an important protective role for the mitochondrial population within cells.

We have also generated mice with conditional alleles of Mfn1 and Mfn2 and are using these mouse lines to examine the role of mitochondrial fusion in adult tissues (Hsiuchen Chen). For example, we have discovered that loss of Mfn2 results in a highly specific degeneration of Purkinje neurons in the cerebellum. These studies are highly relevant to our understanding of several human diseases in which defects in mitochondrial dynamics lead to neurodegeneration (see below). We have also developed mouse models to understand mitochondrial fission (Zhiyin Song and Hsiuchen Chen) and to track mitochondrial dynamics *in vivo* (Anh Pham).

II. 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 (a topic we previously studied). 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 at a similar level of resolution and to determine whether there are common features to these diverse forms of membrane fusion. Mitofusins are the only conserved mitochondrial outer membrane proteins involved in fusion. Therefore, it is likely that they directly mediate membrane

fusion. Consistent with this idea, mitofusins are required on adjacent mitochondria to mediate fusion. In addition, mitofusins form homotypic and heterotypic complexes that are capable of tethering mitochondria. We are trying to determine how tethered mitochondria, mediated by mitofusins, proceeds to full fusion. It should be noted that mitochondrial fusion is likely to be more complicated than most other intracellular membrane fusion events, because four lipid bilayers must be coordinately fused.

In the past year, we used biochemical experiments to show that OPA1 is a lipid-binding GTPase that can deform lipid bilayers (Tadato Ban). We are using similar approaches to understand how mitofusins mediate outer membrane fusion (Prashant Mishra). We are also using biochemical approaches to understand how mitochondrial fission complexes are assembled on the surface of mitochondria (Yan Zhang). These biophysical experiments are complemented by *in vivo* mouse studies (Zhiyin Song and Hsiuchen Chen).

III. 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 that lead to degeneration of axons in peripheral nerves. We have used mouse and cellular models to understand how disease alleles of Mfn2 lead to neuronal dysfunction.

The most common inherited form of optic neuropathy (autosomal dominant optic atrophy) is caused by mutations in OPA1.

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. We are exploring how mitochondrial fusion might affect the segregation of mtDNA species within a cell (Oliver Loson), and how such segregation would affect phenotypic expression of pathogenic mtDNA (Anne Chomyn).

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). In addition, these studies may be relevant to understanding the link between mtDNA mutations, neurodegeneration, and age-related tissue degeneration (Marc Vermulst).

IV. Additional research areas

In addition to mitochondrial dynamics, we have broad interests in other areas of mitochondrial biology. In particular, we have made substantial progress in understanding the organization of mtDNA into nucleoids by solving the structure of DNA bound by TFAM, an mtDNA packaging protein (Huu Ngo). In addition, we are developing tools to analyze mtDNA function and dynamics (Yun Elisabeth Wang).

In collaboration with Caltech's Proteome Exploration Laboratory, we have also developed methods to understand how the mitochondrial proteome changes in response to stress. We are using this approach to study the turnover of mitochondria through autophagy (Nickie Chan), a topic that is relevant for understanding Parkinson's disease.

182. OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation

Tadato Ban, Jurgen A.W. Heymann[*], Zhiyin Song, Jenny E. Hinshaw[*]

The dynamin-related GTPase OPA1 is mutated in autosomal dominant optic atrophy (DOA) (Kjer type), an inherited neuropathy of the retinal ganglion cells. OPA1 is essential for the fusion of the inner mitochondrial membranes, but its mechanism of action remains poorly understood. Here we show that OPA1 has a low basal rate of GTP hydrolysis that is dramatically enhanced by association with liposomes containing negative phospholipids such as cardiolipin. Lipid association triggers assembly of OPA1 into higher order oligomers. In addition, we find that OPA1 can promote the protrusion of lipid tubules from the surface of cardiolipin-containing liposomes. In such lipid protrusions, OPA1 assemblies are observed on the outside of the lipid tubule surface, a protein-membrane topology similar to that of classical dynamins. The membrane tubulation activity of OPA1 is suppressed by GTPgammaS. OPA1 disease alleles associated with DOA display selective defects in several activities, including cardiolipin association, GTP hydrolysis and membrane tubulation. These findings indicate that interaction of OPA1 with membranes can stimulate higher order assembly, enhance GTP hydrolysis and lead to membrane deformation into tubules.

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Publication

Ban, T., Heymann, J.A., Song, Z., Hinshaw, J.E. and Chan, D.C. (2010) OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation. *Hum. Mol. Genet.* **19**:2113-2122.

183. Molecular function of Parkin in mitochondrial quality control

Nickie C. Chan, Michael Sweredoski[1], Natalie Kolawa[2], Robert Graham[1], Sonja Hess[1]

PARK2 is the most commonly mutated gene known to cause autosomal recessive Parkinson's diseases. *PARK2* encodes the ubiquitin E3 ligase Parkin, which is recruited to dysfunctional mitochondria with decreased transmembrane potential. Recent studies proposed that the recruitment of Parkin is responsible for mediating the selective elimination of damaged mitochondria from the cell. This process requires the autophagy machinery and is termed "mitophagy." To date, the molecular mechanism of mitophagy is unresolved. Therefore, it is important to understand how depolarized mitochondria signal Parkin recruitment and to identify the proximal function of Parkin on mitochondria. Such insights will have important implications for the pathogenesis of Parkinson's diseases.

To investigate the role of Parkin on mitochondria, we are employing a quantitative proteomics approach to comprehensively screen for changes in the mitochondrial proteome in response to Parkin recruitment. By combining stable isotope labeling by amino acids in cell culture (SILAC) with mass spectrometry analysis at the Caltech Proteome Exploration Laboratory, we can now routinely identity and quantify close to 80% of the known mitochondrial proteome, by analyzing peptides prepared from isolated mitochondria of cultured HeLa cells. We are also identifying and quantifying an additional 1500-2000 proteins that are associated with the isolated mitochondria.

Using this approach, we have identified key changes in the mitochondrial proteome induced by Parkin recruitment. Besides confirming the changes that have been observed previously, we have also identified a number of novel changes. These changes include a set of mitochondrial proteins that are degraded in a Parkin-dependent manner, as well as the recruitment of a number of non-mitochondrial proteins to mitochondria. We are currently characterizing these proteins with biochemical and cell biological experiments.

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184. Mitochondrial fusion promotes mtDNA stability and tolerance to mtDNA mutations

Hsiuchen Chen, Marc Vermulst, Y. Elisabeth Wang

Mutations in mitochondrial DNA (mtDNA) cause progressive diseases and may be central to the aging process. It is therefore important to identify factors that safeguard mtDNA stability. Mitochondrial fusion is thought to protect cells from dysfunction by enabling exchange of contents, including mtDNA. We have examined the function of mitochondrial fusion in skeletal muscle through the tissue-specific deletion of the mitofusins Mn1 and Mfn2. We showed that mice lacking mitochondrial fusion in muscle have a 14-fold reduction in mtDNA levels and accumulate mtDNA point mutations and deletions. Moreover, these mice have hallmarks of mitochondrial myopathy and die extremely prematurely. We also show that fusion-deficient mice cannot tolerate mutations arising from an error prone mtDNA polymerase. These results indicate that mitochondrial fusion is important for maintaining levels and fidelity of mtDNA. Furthermore, when mtDNA mutations are present, robust mitochondrial fusion is necessary to preserve cellular health. Therefore, mitochondrial fusion is likely a protective factor in aging and mtDNA diseases.

Publications

Chen, H. and Chan, D.C. (2009) Mitochondrial dynamics--fusion, fission, movement, and mitophagy--in neurodegenerative diseases. *Hum. Mol. Genet.* **18**:R169-176.

Chen, H., Vermulst, M., Wang, Y.E., Chomyn, A., Prolla, T.A., McCaffery, J.M. and Chan, D.C. (2010) Mitochondrial fusion is required for mtDNA stability in skeletal muscle and tolerance of mtDNA mutations. *Cell* **141**:280-289.

185. Effect of mitochondrial dynamics on threshold of expression of mutant phenotype in cells carrying a deleterious mtDNA mutation

Anne Chomyn

Human cells have a remarkable ability to tolerate mtDNA mutations without adverse effects on respiration. As an example, a $tRNA^{lys}$ mutation at position 8344 in human mtDNA is responsible for a disease called Myoclonic Epilepsy with Ragged Red Fibers (MERRF) (1). In patient cells, this mutation coexists with wild-type mtDNA, a stated termed heteroplasmy. Studies in cybrids have shown that such heteroplasmic cells have normal respiration levels if the proportion of mutant mtDNA is ≤90% (2). One reason that the threshold for expression of the mutant phenotype (low mitochondrial protein synthesis rate and low respiration rate) is so high is that the mutation is leaky; that is, the mutant tRNA has partial function (3). We believe that another important factor responsible for this high threshold is the ability of mitochondria to fuse and allow mixing of their contents, and thereby allow complementation of the defect.

To test this hypothesis, I will knock down expression of two mitochondrial fusion genes, Mfn1 and Mfn2 by RNA interference in cybrids heteroplasmic for the mutation, and determine whether the threshold for expression of the mutant phenotype is changed.

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186. Mitochondrially targeted restriction enzymes and mitochondrial DNA transfection

Oliver Losón

A major technical gap in mitochondrial research has been the inability to transfect mitochondrial DNA (mtDNA). To address this gap, our strategy is to target restriction enzymes to the mitochondria to set up a selection scheme for mtDNA transfection. When restriction enzymes are targeted to mitochondria, they destroy the organelle's genome, resulting in ρ^0 cells that are devoid of mtDNA. ρ^0 cells are incapable of oxidative phosphorylation and rely solely on glycolysis. Our goal is to transfect a restriction enzyme resistant mtDNA genome into cells expressing a sensitive genome. We hope that this selection scheme will allow re-population of the mitochondria with the transfected genome.

187. Mitochondrial dynamics and the cellular inheritance of mtDNA

Oliver Losón, Anne Chomyn

We are interested in understanding whether mitochondrial dynamics plays a role in the allocation of mtDNA molecules between daughter cells. In some instances, cells and organisms can have more than one type of mtDNA, a condition termed heteroplasmy. Persons suffering from diseases caused by mutations in their mtDNA always have some level of heteroplasmy, where the severity of the disease tends to correlate with the proportion of the mutant haplotype to normal one. Insight into the mechanisms of mtDNA inheritance between cells would shed light on the pathogenic process whereby tissues accumulate mutant mtDNA. To address this issue, we have created cell lines in which the mitofusin proteins (Mfn1 and Mfn2) that mediate fusion between mitochondria can be inducibly deleted, and in which there are two different mtDNA haplotypes present (mouse BALB/c and NZB). Our goal is to monitor the distribution of these mtDNA haplotypes over successive cellular generations and to assess whether mitochondrial dynamics has a role in the partitioning of mtDNA to daughter cells. We have established a quantitative assay for measuring the proportions of these haplotypes, and can reliably measure differences as small as 5%. Currently, we are optimizing the Cre-mediated deletion of the mitofusins in these cell lines. Also, we are establishing and optimizing a single cell PCR assay for measuring the proportions of the mtDNA haplotypes. Pilot experiments have shown that population measurements may not have the sensitivity necessary for monitoring changes in haplotype profiles as a consequence of mitofusin deletion. Thus, establishing the single cell assay is important. In a parallel line of experiments, we have generated a mouse model that is both heteroplasmic for mtDNA and conditional for mitofusins.

188. Mechanisms and regulation of mitochondrial fusion

Prashant Mishra

Recent data indicates that mitochondria undergo dramatic dynamic behaviors within a cell, including biogenesis, targeted destruction, fission, and fusion with other mitochondria. Mitochondrial fusion has been shown to play an important role in mammalian biology on numerous levels, from proper functioning of the organelle, to cellular homeostasis, to organismal development and functioning. Fusion appears to be mediated and regulated by the mitofusin proteins, members of the dynamin-like family of large GTPases that are specifically involved in membrane remodeling events. We are attempting to reconstitute the mitochondrial fusion process *in vitro*, in order to elucidate the roles of individual components. Expression and purification of recombinant mitofusins, followed by incorporation into liposomes, allows us to study the activity of these molecules in an isolated membrane bilayer. In a parallel approach, mitochondria are isolated from mammalian cells and the fusion capacity of these organelles is studied in an *in vitro* setting. With these assays, we hope to elucidate molecular details of the mitochondrial fusion process, as well as screen cytosolic preparations for regulatory components.

189. Crystal structure of human TFAM, an mtDNA packaging protein, bound to DNA

*Huu Ngo, Nickie Chan, Jens Kaiser**

Mitochondria contain a circular genome that is organized into compact protein/DNA structures called nucleoids. Mitochondrial transcription factor A (TFAM) is a major component of such nucleoids and is essential for mtDNA maintenance and packaging. TFAM is a member of the high mobility group (HMG) family of DNA-binding proteins, which cause a kink in DNA upon binding. When bound to naked DNA, TFAM induces its compaction into nucleoid-like structures. Protein measurements in human cells indicate that TFAM is abundant enough, relative to mtDNA, to wrap mtDNA genomes into nucleoid structures. On average, there are about 1000 TFAM molecules for 1 mtDNA genome. Therefore, it has been suggested that TFAM functions analogously to histones in the eukaryotic nucleus or to the HU protein in bacteria. Little structural information is known about how TFAM interacts with mtDNA to form a compact nucleoid structure. In this study, we have solved a 2.4 Å crystal structure of the TFAM-DNA complex. The complex crystallized in the space group C2221 with the following unit cell parameters: a = 68.44, b = 81.91, and c = 161.25 Å. One protein molecule is bound to the DNA duplex in the asymmetric unit. Experimental phases were obtained using MAD methods with co-crystals of selenomethionine-substituted TFAM and DNA. The initial model was built into the 2.4 Å MAD experimental maps using COOT and refined using PHENIX. The structure of the complex reveals two tandem HMG boxes binding to the minor-groove of DNA and bending the DNA into a U. Each HMG domain has three helices folded in an L-shape

configuration that intercalates into the DNA. The HMG boxes make H-bonds with several bases of the DNA duplex. In addition to the HMG domains, the structure also exhibits an α-helical linker with positively charged residues that interact with the DNA phosphate groups to stabilize the severe DNA bend.

190. Generation and characterization of an inducible Cre reporter for monitoring mitochondrial dynamics

Anh Pham

Mitochondria are dynamic organelles that undergo continuous cycles of membrane fusion and scission. These dynamic properties are important for maintaining mitochondrial DNA content, oxidative respiration, number and distribution. In particular, genes regulating mitochondrial morphology have been associated with many neurodegenerative diseases, including Charcot-Marie Tooth 2A and Parkinson's disease. Gene knockout studies in mice have also shown that perturbations to the fusion or fission machinery--resulting in fragmented or long interconnected tubules, respectively--cause embryonic lethality. To explore the molecular mechanisms of mitochondrial dynamics *in vivo* and in tissues, we have designed a mouse line (ROSA26-mito-Dendra2$^{floxed\text{-}stop}$) with an inducible Cre reporter that labels mitochondria with a photoconvertible fluorescent protein, Dendra2. This mouse line provides several advantages for assessing mitochondrial dynamics in live tissues. First, the photoconvertible properties of Dendra2, which converts from green to red emission with induction of UV light, permits monitoring of individual organelles and quantification of mitochondrial fusion events by the exchange of photoconverted signal. Secondly, the inducible design enables cell-specific labeling of mitochondria within a heterogeneous cellular milieu. We have now performed immunohistochemical analysis of various tissues from this line and confirmed the faithful induction of mito-Dendra2 in the presence of Cre expression. Moreover, primary cells isolated from cardiac and skeletal muscles of these animals demonstrate appropriate localization of Dendra2 to the mitochondrial matrix. Importantly, we have observed no adverse effects in fertility or lifespan of animals carrying the homozygous and heterozygous alleles of the floxed or induced transgene. In summary, we have generated a versatile mouse line that can be used to characterize mitochondrial phenotypes in novel transgenic lines or to investigate the physiology of mitochondrial fusion and fission in tissues.

191. Screen for genes that involved in mitochondrial morphology and synaptic localization

Anna Salazar

Mitochondria are extraordinarily dynamic organelles. They are involved in numerous pathways, such as the cell cycle, cell growth, cell death, cell signaling, and cellular differentiation. They undergo fusion, fission and trafficking throughout the cell along microtubule networks. Mitochondria are also involved in several human diseases, such as various mitochondrial DNA (mtDNA) diseases, Parkinson's disease, and Charcot-Marie Tooth type 2A. The latter disease causes degeneration of axons to the lower extremities, creating muscle degeneration and foot/gait abnormalities.

One model system that can be used to investigate such diseases, including the underlying mitochondrial dynamics and trafficking that may underlie the diseased state, is *Drosophila melanogaster*. *Drosophila* provide a powerful system in which to investigate many basic science questions and to study human diseases, with flies containing a homologue of most human disease genes. Furthermore, the powerful genetic tools currently available, as well as the one-week generation time, combine to make the fruit fly into an ideal model system.

Mitochondria are normally transported along the central nervous system axons out to the *Drosophila* larval neural muscular junction where they are found along the axons and at boutons, the parts axonal specializations that makes a synaptic connection with the muscle. Genes that alter mitochondrial morphology and/or trafficking can result in a decrease in mitochondria at more distal axons and a decrease in mitochondria at synapses. We will be conducting a directed screen of genes that encode proteins localized to the mitochondria, as assessed by mass spectrometry. We will be using UAS-RNAi lines, which are fly lines in which one gene is silenced through targeting the RNA of that gene for tissue specific degradation. We will be looking for mitochondrial morphological changes and localization differences, in order to screen for genes that perturb normal mitochondrial function or structure, or may interfere in normal mitochondrial dynamics. We will also be looking for axonal and synaptic differences, in general, which may occur in spite of a lack of gross morphological changes and may help in the understanding of disease. We will use genetics, biochemistry, and cell biology to further understand the mechanism of action of these genes of interest.

192. The role of Mff in mitochondrial fission

Zhiyin Song, Hsiuchen Chen

Mitochondria in living cells form dynamic tubular networks that undergo continuous fission and fusion events. Mfn1, Mfn2 and OPA1 are central to the fusion of mammalian mitochondria; Drp1, Fis1 and Mff are central to their fission. Drp1 (Dnm1p in yeast), a member of the dynamin family of large GTPases, has two important domains including an N-terminal GTPase domain and a C-terminal GTPase effector domain (GED). Drp1 is located mostly in the cytosol of mammalian cells but translocates to mitochondria upon induction of mitochondrial fission. Mff is a mitochondrial outer membrane protein with a transmembrane domain (TM) at its C-terminal. Knockdown of Mff in mammalian cells results in the elongation of mitochondria (1).

To analyze the function of Mff in mitochondrial dynamics, we generated Mff null cell lines from mice with

a mutation in the Mff gene. We found that Mff null cells have substantially longer tubular mitochondria that undergo fewer mitochondrial fission events than in WT cells. In addition, we found that Mff null cells have longer peroxisomes, consistent with the previous study (1). We are currently using these cells to elucidate the mechanism of Mff function in mitochondrial fission.

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193. Structural and functional studies of the mitochondrial fission complex

Yan Zhang

Fission is an important feature of mitochondria, an essential organelle of eukaryotic cells. Balanced by fusion, mitochondrial fission controls mitochondrial size, shape, distribution, and function. Several studies have demonstrated that mitochondrial fission may function early during apoptosis and serve an important role in the cell death machinery. In addition, mitochondrial fission is important for proper mitochondrial distribution in neurons, and has been linked to cellular senescence. In human, lack of mitochondrial fission results in neonatal lethality. Therefore, it is imperative to understand the molecular mechanism of mitochondrial fission.

At the mechanistic level, mitochondrial fission is best understood in the budding yeast *S. cerevisiae*. The central player, the dynamin-like protein Dnm1p, self-assembles into spiral and ring structures in a nucleotide-dependent manner. The recruitment of Dnm1p depends on an integral mitochondrial outer membrane protein Fis1p and the molecular adaptor proteins Mdv1p and Caf4p. Mdv1p and Caf4p are homologous, soluble proteins containing an NH2-terminal extension (NTE), a coiled-coil (CC) region, and a COOH-terminal seven-WD repeat domain. Acting as molecular bridges, these proteins bind to Fis1p through the NTE region and to Dnm1p through the WD40 region. Moreover, Mdv1p and Caf4p are capable of forming homotypic and heterotypic interactions probably through interactions in the CC domain.

A central issue in mitochondrial dynamics is how fission complexes are recruited and activated. To mechanistically understand how oligomerization state of the fission complex is controlled, we are using X-ray crystallography and cell biology approaches to study the oligomerized Fis1p/Mdv1p NTE-CC complex. We have expressed, purified and crystallized these recombinant protein complexes. After extensive optimization, the crystals diffracted to 4.0Å with severe anisotropy. To improve diffraction quality, we mutated two residues between the NTE and the CC domain of Mdv1p identified through the Surface Entropy Reduction Prediction Server. Crystals of these mutants show improved diffraction ability to 3.7Å with moderate anisotropy. Molecular replacement with the known structure of Fis1p, however, has not been successful. Currently, these crystals are under further optimization. We are also producing selenomethionine labeled complexes for phasing.

194. Mitochondrial dynamics and mtDNA nucleoids

Yun Elisabeth Wang

Disruption of mitochondrial fusion decreases membrane potential and respiratory capacity, while greatly increasing the number of defective mitochondria lacking mitochondrial DNA-containing nucleoids. It is also well known that both changes in mitochondrial dynamics and changes in mitochondrial DNA stability are correlated with neurodegenerative disease. Recently, we found that loss of either both mitofusins Mfn1 and Mfn2 or of the fusion protein OPA1 leads to a concurrent severe decrease in mtDNA copy number. This suggests that mitochondrial dynamics plays an important role in maintaining mtDNA copy number and that loss of mtDNA may contribute to the mitochondrial dysfunction seen in neurodegenerative disease.

To further examine the interdependence between mitochondrial dynamics and mtDNA stability, we have developed a novel system that allows for simultaneous live-cell tracking of mtDNA nucleoids and of mitochondria. We are using this system to characterize mtDNA nucleoid dynamics with respect to mitochondrial dynamics. Specifically, we will investigate the role of mitochondrial fusion on mtDNA nucleoid dynamics.

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Ban, T., Heymann, J.A., Song, Z., Hinshaw, J.E. and Chan, D.C. (2010) OPA1 disease alleles causing dominant optic atrophy have defects in cardiolipin-stimulated GTP hydrolysis and membrane tubulation. *Hum. Mol. Genet.* **19**:2113-2122.

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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 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 300 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 350 members, due to combinatorial mixing of subunits. Thus, there may be as many as 650 distinct RING ligase complexes, which would make it the largest-known family of enzymes in human cells (*Petroski and Deshaies, 2005a*; Deshaies and Joazeiro, 2009). 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 sensing of glucose. 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, 2005b*).

We are now using a variety of assays that employ reconstituted SCF to address basic questions that are of central importance to understanding the mechanisms that underlie the operation of the ubiquitin-proteasome system (UPS). Although we have made considerable progress over the past year in understanding how SCF works (see abstracts by G. Kleiger, N. Pierce, and A. Saha, as well as Saha and Deshaies, 2009), much remains to be done. For example, we still do not have answers to the following fundamental questions: 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? What is the full range of mechanisms that enable processive ubiquitination? 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, 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 an intrinsic 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 SCFCdc4 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. We are currently seeking to extend these studies by developing a purified substrate bearing a ubiquitin chain of defined length and linkage. The objective will be to use a chemically-defined substrate to initiate quantitative kinetic studies on the mechanism of action of the proteasome (see abstract by M. Rome).

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. As part of the effort, we are investigating how proteasome inhibitors bring about elevated synthesis of proteasome subunits in mammalian cells (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. In collaboration with Tim Lewis at the Broad Institute, a set of ubistatin-like derivatives has been produced and we plan to investigate these new compounds to identify ubistatin-like molecules that efficiently block protein turnover within 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 (known as p97 in human cells), 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 at least thirteen distinct p97 complexes in human cells (see abstract by G. Alexandru and Alexandru *et al.*, 2008). 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 in yeast cells (see abstract by R. Verma) or hypoxia-inducible factor-1a in mammalian cells (Alexandru *et al.*, 2008; see abstract by W. den Besten). 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: Complex mixtures of proteins can be analyzed directly by trypsinization followed by multidimensional chromatography and mass spectrometry to characterize their protein composition. One such method, known as MudPIT (for multidimensional protein identification technology) was employed by Johannes Graumann and Thibault Mayor in our lab to study 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 in whole cell extracts or in chromatin fractions may enable us to see subtle defects, such as those that occur when one member of a redundant pair of enzymes is mutated (see abstracts by N. Kolawa and K.-J. Chang).

In a second project, we plan to use crosslinking and stable isotope labeling to track 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. During the past year, we established that the MEN enables Cdc14 to be relocated to the cytoplasm during late anaphase by phosphorylating and inactivating a nuclear localization sequence near the C-terminus of Cdc14 (see abstract by D. Mohl and Mohl *et al*., 2009). This causes Cdc14 to accumulate in the cytoplasm, where it dephosphorylates and activates proteins that help drive the cell out of mitosis and into the subsequent G1 phase. We now plan to address how the MEN dislodges Cdc14 from Net1 to initiate the process of Cdc14 activation.

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195. p97 regulation via interaction with UBX domain-containing co-factors

Gabriela Alexandru

p97/Cdc48 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 protein 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 substrate-recruiting 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 for p97's diverse functions we have analyzed p97 immunoprecipitates from human tissue culture cells by MudPIT (Multidimensional Protein Identification Technology), searching for new p97 co-factors (Alexandru *et al.*, 2008). This analysis revealed eight p97 binding partners, all having a UBX domain in their C-terminal region. 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 Flag-UBX protein immunoprecipitates from human cells revealed that UBA-UBX proteins bind ubiquitinated proteins and also interact with multiple E3 ubiquitin ligases, suggesting they might be involved in ubiquitin-dependent protein degradation. This analysis was initially aimed to identify substrates interacting specifically with each UBX-domain co-factor of p97. In particular, we found that UBXD7 mediated p97 interaction with the CUL2/VHL ubiquitin ligase and its substrate, the hypoxia-inducible factor 1a (HIF1a). Depletion of p97 by siRNA led to accumulation of endogenous HIF1a and increased expression of the HIF1a target carbonic anhydrase IX. Thereby our work revealed an unexpected role for p97 in functional regulation of HIF1a, which is the key governor of cellular and organismal responses to oxygen tension. Further UBX protein – substrate pairs will be identified and analyzed to better define the role of UBX proteins within the p97 network.

196. Identifying UPS players and their mechanisms in repairing UV-damaged chromosome

Kuang-Jung Chang

The ubiquitin-proteasome system (UPS) is the major pathway for the degradation of protein to regulate protein turnover and antigenic-peptide generation. Recently, it has been shown that it is also involved in regulating gene transcription and DNA repair. Formation of pyrimidine dimers, which is caused by UV irradiation of DNA, triggers nucleotide excision repair to remove the damaged region. However, if pyrimidine dimers in the transcribed strand are not repaired, they can stall RNA polymerase during transcription. This results in activation of the transcription-coupled repair (TCR) pathway to repair the damage and enable transcription to proceed. It has also been shown that stalled RNA polymerase can also be degraded via the UPS; however, the underlying mechanism and how it relates to TCR remains poorly understood. The aim of this project focuses on identifying the connections between UV damaged DNA and the UPS. Our approach involves collecting all of the chromosome binding proteins with or without UV damage by CsCl gradient. Then we use the SILAC (stable isotope labeling with amino acids in cell culture)-quantification mass spectrometry to identify the proteins that are specifically recruited to chromatin by UV. The results of these efforts will help us to begin dissecting the mechanisms and acquire a better understanding of how the UPS relates to transcription-coupled repair.

197. Identification of inhibitors and substrates 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 inhibitors for Cdc48/p97 based on its X-ray structure, we first searched for the scaffolds that are likely to bind to the D2 domain of p97. Of particular interest to us are scaffolds that include an electrophile that can readily react with a natural cysteine near the active site of p97. We have developed several cell-based assays to evaluate whether *in vitro* inhibitors would affect p97-dependent reporter substrates in cells but not the p97-independent substrates. The studies we have performed so far have established that it is feasible to identify specific and reasonably potent inhibitors of p97 that inhibit UPS activity in tissue culture cells. We have finished a larger-scale HTS effort to identify different p97 inhibitors and carried out structure-activity relationship study of the lead compounds. We have generated reversible p97 inhibitors with submicromolar IC_{50} *in vitro* and in inhibiting p97-dependent substrate degradation in cells but not p97-independent substrates. We will carry out X-ray crystal structure study of an inhibitor bound p97 to facilitate design of a higher potency inhibitor with better specificity for future cell culture and *in vivo* studies aimed at evaluating whether p97 is a good target in cancer.

We are employing a high-throughput proteomic approach by using automated fluorescence microscope and fluorescence-activated cell sorter to identify substrates of the Cdc48-pathway in budding yeast. We are screening a yeast library of 4,159 strains, each of which expresses a GFP-tagged ORF enables us to measure the abundance of different yeast proteins. GFP-ORFs accumulated in cells lacking p97/Cdc48 activity are likely to be substrates of p97/Cdc48. The homologous mammalian substrates will be validated by p97 siRNA. Specific inhibitors and substrates of p97 will serve as valuable biological tools for study the physiological function and mechanism of Cdc48/p97.

198. The role of p97 and UBXD7 in the ubiquitination and degradation of HIF-1

Willem den Besten

The abundant hexameric AAA ATPase p97 participates in a wide range of cellular processes and for many of these different pathways, the function of p97 involves the recognition of ubiquitinated protein substrates via specific substrate-recruiting cofactors. The largest group of p97 cofactors consists of proteins that interact with the N-terminus of p97 through a so-called ubiquitin regulatory X (UBX) domain. There are at least 13 UBX proteins encoded in the human genome, five of which also have an ubiquitin-binding (UBA) domain and interact with polyubiquitin conjugates.

P97 has been proposed to function as a "separase," disassembling protein complexes through the application of mechanical force generated by the hydrolysis of ATP. However, studying the mechanism of p97 function in detail has remained difficult because for almost all the UBX adaptors soluble substrates have not yet been discovered. Recent proteomic analysis of the network of p97 co-factors revealed that in addition to binding ubiquitin conjugates, UBA-UBX proteins also interact with dozens of ubiquitin E3 ligases (Alexandru *et al*., 2008). This was especially striking for UBXD7, which binds to many of the components of cullin–Ring E3 ligase (CRL) complexes, and has the strongest binding preference for Cullin2. In addition, UBXD7 links p97 to the E3 ligase complex Cul2/VHL and its substrate Hypoxia-inducible factor 1 alpha (HIF-1α).

The identification of HIF-1α as the first known substrate for UBXD7 opens the door for more detailed analysis of p97 and UBX protein function in the postubiquitination/pre-degradation step of proteolysis. Therefore, by using HIF-1a degradation as a model system, we are looking to answer the following questions:

1. What determines the association between UBXD7 and CRLs?
2. What is the role of UBXD7/p97 complex in the degradation of HIF-1α?
3. What other substrates require the p97 pathway for their efficient proteolysis?

199. 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. Hundreds of proteins within the cell are thought to be targeted for degradation by SCF complexes. Thus, inappropriate regulation of SCF activity would be expected to have multiple negative impacts on cellular homeostasis. Despite impressive advancements in our knowledge about the mechanism of action of SCF complexes, we still know relatively little about how these enzymes are independently regulated. 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 conjugation of the ubiquitin-like protein Nedd8 to the SCF subunit Cul1 by Ubc12 (referred to as 'neddylation'), or negatively regulates Cul1 deneddylation by the COP9 signalosome. Neddylation of Cul1 is necessary for SCF ubiquitin ligase activity and constitutes a key mechanism 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 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 a player in several human malignancies.

200. Binding of 26S proteasome subunits to ubiquitin receptor proteins

Tara Gomez

The proteasome, is a sophisticated proteolytic machine that degrades ubiquitinated proteins. However, 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 the budding yeast, *Saccharomyces cerevisiae*, the base is composed of about eight proteins, one of which, Rpn1 is thought to play an important role in binding to ubiquitin receptor proteins such as Rad23, Dsk2 and Ddi1, all of which contain a ubiquitin-like domain (UBL). It is believed that these UBL proteins deliver ubiquitinated substrates to the proteasome to promote degradation, and may bind the same or overlapping sites of RPN1 via their UBL domain. Using a combination of genetic and biochemical approaches, "hot spot" regions of Rpn1 that limit binding to UBL receptors are being evaluated. These proteasome *cis* mutants can then themselves be used as tools for studying the specific substrate repertoire of various UBL proteins and for elucidating redundancy pathways between various receptors.

201. 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 have hypothesized that key switches governing differentiation pathways are also controlled by the UPS. We have tested this hypothesis by seeking UPS genes that influence embryonic stem cell differentiation into cardiovascular tissue. Our approach has involved the generation of mouse embryonic stem cell lines that express lineage-specific reporter genes, transfecting these cells with a siRNA library, and screening for spontaneous or accelerated differentiation. Preliminary results of our screen suggest a role for several UPS genes; however, we are focusing our efforts on two that stimulate the formation of cardiac progenitor cells. A combination of *in vivo* and *in vitro* studies are being pursued to further delineate the role of these genes.

202. Dynamics and structure of the Cdc34-SCF interaction

Gary Kleiger

Protein degradation by the ubiquitin proteasome system (UPS) is fundamental to cellular homeostasis. Degradation requires assembly of a polyubiquitin chain upon substrate, which is thought to occur in a processive manner. However, the structural and mechanistic features that enable template-independent processivity of ubiquitylation enzymes are unknown. We show that chain assembly by ubiquitin ligase SCF and ubiquitin conjugating enzyme Cdc34 is facilitated by the unusual nature of Cdc34–SCF transactions: Cdc34 binds SCF with nanomolar affinity, nevertheless the complex is extremely dynamic. These incongruent properties are enabled by rapid bimolecular association driven by electrostatic interactions between the acidic tail of Cdc34 and a basic 'canyon' in the Cul1 subunit of SCF. *De novo* docking between Cdc34 and Cul1 predicts an extensive molecular interface between the tail and the basic canyon, an arrangement confirmed by cross-linking and kinetic analysis of acidic tail and basic canyon mutants. Residues in this region on Cul1 are conserved in both Cul1 paralogs and orthologs, suggesting that the same mechanism underlies processivity for all cullin-RING ubiquitin ligases (CRLs).

203. Identifying substrates of the proteasome using quantitative mass spectrometry

Natalie Kolawa

The complexity of ubiquitin proteasome system (UPS) has made it difficult to study. As such, there are still many substrates yet to be discovered and for many proteins there is still no consensus on whether they are true substrates of the UPS.

In the last couple of years, there have been major advances in shotgun mass spectrometry techniques and data analysis software that have enabled quantitative analysis of changes in protein level on a proteome-wide scale. Our goal is to use these new technologies, coupled with stable isotope labeling of amino acids in cell culture (SILAC) to identify and quantify known and novel substrates of the proteasome in yeast on a global scale. This work will establish a catalog of high-confidence substrates and will better elucidate the cellular pathways that the UPS is involved with, allowing for a clearer understanding of the breadth of the UPS's role in the cell.

204. Proteome-wide protein identification facilitated by *in vivo* cross-linking

J. Eugene Lee

Aberrant protein-protein interactions are implicated in a number of human diseases, and understanding protein interaction networks has become the subject of intense research in diverse disciplines. Yet, global detection of protein interactions within biological systems poses a significant challenge for current technology. For instance, mass spectrometry combined with tandem affinity purification is a useful tool for investigating protein interactions on a global scale, but this technique fails to recover transient/weak protein interactions - these interactions are lost during the tandem affinity purification steps. I propose to overcome this problem by introducing a chemical cross-linking strategy aimed at preserving transient protein complexes *in vivo*. Using this strategy, I will attempt to understand the molecular basis for BRCA1, a critical tumor suppressor in the etiology of breast cancer. BRCA1 encodes a ubiquitin ligase, whose enzymatic activity is abolished by mutations found in breast cancer patients. Elucidation of the cellular function of BRCA1 will greatly enhance our knowledge on breast cancer, but our understanding on BRCA1 function is still incomplete. It is necessary to identify the substrates ubiquitinated by BRCA1 and cellular consequences of their ubiquitin modification in order to define the cellular function of BRCA1.

205. Integrated dynamics of Sic1

Nathan Pierce

Attachment of a polyubiquitin chain with at least four ubiquitins targets proteins to the proteasome for degradation. A cascade of three enzymes carries out the synthesis of polyubiquitin chains: an ubiquitin activating enzyme (E1), an ubiquitin conjugating enzyme (E2), and an ubiquitin ligase (E3). RING (really interesting new gene) E3s can processively catalyze the direct transfer of ubiquitin from an E2 to a lysine on a target protein. The pathway by which ubiquitin chains are generated on substrate remains unclear and multiple distinct models involving chain assembly on E2 or substrate have been proposed. However, the speed and complexity of the reaction have prevented direct experimental tests to distinguish between potential pathways. We have developed new theoretical and experimental methodologies to address both limitations. A quantitative framework based on product distribution predicts that the E3 SCF^{Cdc4} and the E2 Cdc34 build polyubiquitin chains on substrates by sequential transfers of single ubiquitins. Direct measurements with millisecond time resolution carried out on a quench-flow device demonstrate that substrate polyubiquitylation proceeds sequentially. A quantitative model of the processive reaction yield

individual rate constants for substrate dissociation and ubiquitin transfer at each step of chain assembly. Our results present an unprecedented glimpse into the mechanism of RING ubiquitin ligases and provide a universal framework from which the complete mechanistic dissection of RING ubiquitin ligases is possible.

206. Understanding and targeting the proteasome recovery pathway in cancer

Senthil K. Radhakrishnan

In *Saccharomyces cerevisiae*, chemical or genetic inhibition of proteasome activity induces new proteasome synthesis promoted by the transcription factor RPN4. This ensures that proteasome activity is matched to demand. This transcriptional feedback loop is conserved in mammals, but its molecular basis had remained unclear. Recently, we discovered that nuclear factor erythroid-derived 2-related factor 1 (Nrf1), a transcription factor of the cap "n" collar basic leucine zipper family, but not the related Nrf2, is necessary for induced proteasome gene transcription in mouse embryonic fibroblasts (MEFs) (Radhakrishnan *et al*., 2010). Promoter-reporter assays revealed the importance of antioxidant response elements in Nrf1-mediated upregulation of proteasome subunit genes. $Nrf1^{-/-}$ MEFs were impaired in the recovery of proteasome activity after transient treatment with the covalent proteasome inhibitor YU101, and knockdown of Nrf1 in human cancer cells enhanced cell killing by YU101. Taken together, our results suggest that Nrf1-mediated proteasome recovery pathway could be an attractive target for therapeutic intervention in cancer. Currently, we are investigating if this recovery pathway could be blocked by small molecules. Overall, our approach could lead to rational drug combinations and hence effective therapies against cancer.

Publication

Radhakrishnan, S.K., Lee, C.S., Young, P., Beskow, A., Chan, J.Y. and Deshaies, R.J. (2010) Transcription factor Nrf1 mediates the proteasome recovery pathway after proteasome inhibition in mammalian cells. *Mol. Cell* **38**:17-28.

207. Assembly and disassembly of cullin-RING ubiquitin ligases

Michael Rome

Cullin-RING ubiquitin ligases (CRLs) comprise a major family of E3 ubiquitin ligases that mediate the turnover of a diverse repertoire of cellular proteins. CRLs covalently modify substrates by building ubiquitin chains on target lysine residues, which often result in the destruction of ubiquitinated proteins by the 26S proteasome. CRLs must assemble into active ubiquitin ligase complexes, which require the association of at least six independent protein co-factors to mediate substrate ubiquitination. In addition, the regulation of CRL complexes is a highly dynamic process, as multiple effector proteins associate with CRLs in order to facilitate or inhibit the process of ubiquitination. Two key regulators of CRLs, the COP9 signalosome and Cand1, are thought to play inhibitory roles by disassembling and inhibiting active ubiquitin ligase complexes. Using *in vitro* biochemistry and mechanistic enzymology, I propose to study the effect of Cand1 and COP9 on CRL assembly and disassembly. To do this, I am currently building quantitative fluorescence assays to study Cand1 and COP9 dynamics. In addition, I will combine classical biochemical approaches with mechanistic enzymology in order to understand how CRLs are regulated *in vivo*.

208. Mechanism of substrate ubiquitination and degradation by ubiquitin-proteasome system

Anjanabha Saha

Protein turnover by the ubiquitin-proteasome system (UPS) involves substrate ubiquitination by ubiquitin conjugation machinery followed by degradation by the 26S proteasome. Protein turnover is often regulated at the level of substrate ubiquitination by cullin-RING ligases (CRLs). We are utilizing the human SCF complex, the prototype of CRLs, to investigate the mechanism of ubiquitination of substrates like p27 and IkB. In addition, we are examining how reversible modification of CRLs by neddylation stimulates substrate ubiquitination. Current studies indicate that neddylation imparts conformational flexibility within the SCF, which brings the thioesterified E2 and the bound substrate in close proximity enabling increased rate of ubiquitin transfer (Saha and Deshaies, 2008). Additionally, we are investigating the mechanism of degradation of ubiquitinated substrates (p27 and IkB) and role of additional factors involved in substrate turnover.

209. 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. 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, and is the preferred receptor pathway for the S-Cdk inhibitor Sic1. However, Rpn10 is dispensable for the degradation of substrates such as misfolded CPY*. Instead, the Ub-selective chaperone Cdc48 and its adaptors are needed for degradation of CPY*.

Cdc48/p97 is an essential ATPase whose role in targeting substrates to the UPS is unclear. Existing models posit that Cdc48 acts upstream of UPS receptors. To address this hypothesis, we have examined the association of Ub conjugates with 26S proteasomes. Surprisingly,

proteasomes isolated from *cdc48-3* contain high levels of Ub conjugates, equivalent to peptidase-inhibited proteasomes from wildtype cells. Shotgun mass spectrometry identified numerous non-proteasomal proteins (PIPs), which are enriched in proteasomes isolated from *cdc48-3*. The turnover of two PIPs: Hsl1, a checkpoint kinase and Rpb1, the largest subunit of RNA Pol II was further characterized. Cell cycle-dependent proteolysis of the former was indeed dependent on Cdc48. UV-induced turnover of Rpb1 depended upon both Cdc48 and its uncharacterized adaptor, Ubx5. Cdc48, proteasomes, and ubiquitinated Rpb1 accumulate on chromatin in UV-treated cells and the latter two accumulate to even higher levels in *cdc48-3* cells, suggesting that degradation of ubiquitinated Rpb1 is facilitated by Cdc48 at sites of stalled transcription. Our data is beginning to reveal a hitherto unappreciated coupling of function between proteasomes and Cdc48 that we suggest is necessary to sustain processive degradation of unstable multidomain subunits of large protein complexes.

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Summary: Our laboratory has been generally interested in how cells proceed through the cell cycle in an orderly manner. In order to undergo division, cells must replicate their DNA during S-phase and then distribute the duplicated copies of their genomes equally to daughter cells at M-phase or mitosis. In earlier years, we focused mainly on the enzymatic network that induces the entry of cells into mitosis. A master regulatory kinase called MPF triggers mitotic entry by phosphorylating a myriad of cellular proteins. These phosphorylations lead to the hallmark events of mitosis such as chromosome condensation, nuclear envelope disassembly, and assembly of the mitotic spindle. MPF, which stands for maturation- or mitosis-promoting factor, is a heterotrimer containing a cyclin, a cyclin-dependent kinase (Cdk), and a small ancillary protein Cks protein. The kinase subunit of MPF is Cdk1, the founding member of this family--it was historically known as Cdc2. MPF also typically contains one of the B-type cyclins.

In order for MPF to induce mitosis, it is essential that prior events in the cell cycle have occurred normally. Notably, the cell must have copied all of its genomic DNA accurately during S-phase. In addition, the DNA must also be free of damage in order for the cell to begin division. If a cell has not replicated its DNA accurately or has suffered damage in the genome, various checkpoint mechanisms impose a blockade to mitotic entry. This delay allows time for the cell to repair DNA lesions. These checkpoint responses have additional physiological consequences. For example, these pathways can influence the transcriptional program of the cell, help to stabilize aberrantly stalled replication forks, and participate in the decision to engage in apoptosis in the event of very severe damage.

Checkpoint pathways consist of sensor proteins that detect problems with the DNA and effector proteins that, for example, regulate the function of cell cycle control proteins. Various mediator proteins manage interactions between sensor and effector proteins in order to control the specificity and efficiency of checkpoint pathways. In cells with incompletely replicated DNA, a master regulatory kinase known as ATR functions near the apex of the checkpoint pathway. The action of ATR ultimately leads to the activation of a downstream effector kinase known as Chk1. A distinct kinase called ATM becomes activated in cells with various forms of damaged DNA, such as DNA with double-stranded breaks (DSBs). Both ATR and ATM are members of the phosphoinositide kinase-related family of protein kinases (PIKKs).

Much of our work now involves a study of the molecular pathways that lead to the activation of ATR. We are also interested in the targets of this kinase and the roles of these targets in checkpoint responses. In recent years, we have found that the activation of ATR occurs through interaction with a specific activator protein called TopBP1. We have also identified a novel mediator protein called Claspin that enables activated ATR to recognize and phosphorylate Chk1. We are now pursuing a thorough characterization of this pathway in order to elucidate new players and regulatory principles. These studies should eventually help us understand how cells maintain the integrity of their genomes. This issue is very relevant to human health because an overarching problem with cancer cells is that such cells have suffered a catastrophic deterioration in the mechanisms that maintain genomic stability.

210. 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.

211. 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.

212. 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.

213. 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.

214. The MRN complex mediates activation of TopBP1 by ATM

Hae Yong Yoo, Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

The activation of ATR-ATRIP in response to double-stranded DNA breaks (DSBs) depends upon ATM in human cells and *Xenopus* egg extracts. One important aspect of this dependency involves regulation of TopBP1 by ATM. In *Xenopus* egg extracts, ATM associates with TopBP1 and thereupon phosphorylates it on S1131. This phosphorylation enhances the capacity of TopBP1 to activate the ATR-ATRIP complex. We show that TopBP1 also interacts with the Mre11-Rad50-Nbs1 (MRN) complex in egg extracts in a checkpoint-regulated manner. This interaction involves the Nbs1 subunit of the complex. ATM can no longer interact with TopBP1 in Nbs1-depleted egg extracts, which suggests that the MRN complex helps to bridge ATM and TopBP1 together. The association between TopBP1 and Nbs1 involves the first pair of BRCT repeats in TopBP1. In addition, the two tandem BRCT repeats of Nbs1 are required for this binding. Functional studies with mutated forms of TopBP1 and Nbs1 suggested that the BRCT-dependent association of these proteins is critical for a normal checkpoint response to DSBs. These findings suggest that the MRN complex is a crucial mediator in the process whereby ATM promotes the TopBP1-dependent activation of ATR-ATRIP in response to DSBs.

215. Treslin collaborates with TopBP1 in triggering the initiation of DNA replication

Akiko Kumagai, Anna Shevchenko, Andrej Shevchenko, William G. Dunphy

TopBP1 has important roles in both DNA replication and checkpoint regulation in vertebrates. We have identified a protein called Treslin that associates with TopBP1 in *Xenopus* egg extracts. Depletion of Treslin from egg extracts strongly inhibits chromosomal DNA replication. Binding of Treslin to chromatin in egg extracts occurs independently of TopBP1. However, loading of the initiator protein Cdc45 onto chromatin cannot take place in the absence of Treslin. Prior to the initiation of DNA replication, Treslin associates with TopBP1 in a Cdk2-dependent manner. Ablation of Treslin from human cells also strongly inhibits DNA replication. Taken together, these results indicate that Treslin and TopBP1 collaborate in the Cdk2-mediated loading of Cdc45 onto replication origins. Thus, Treslin regulates a pivotal step in the initiation of DNA replication in vertebrates.

216. Rad17 plays a central role in establishment of the interaction between TopBP1 and the Rad9-Hus1-Rad1 complex at stalled replication forks

Joon Lee, William G. Dunphy

Rad17 is critical for the ATR-dependent activation of Chk1 during checkpoint responses. It is known that Rad17 loads the Rad9-Hus1-Rad1 (9-1-1) complex onto DNA. We show that Rad17 also mediates the interaction of 9-1-1 with the ATR-activating protein TopBP1 in *Xenopus* egg extracts. Studies with Rad17 mutants indicate that binding of ATP to Rad17 is essential for the association of 9-1-1 and TopBP1. Furthermore, hydrolysis of ATP by Rad17 is necessary for the loading of 9-1-1 onto DNA and the elevated, checkpoint-dependent accumulation of TopBP1 on chromatin. Significantly, a mutant 9-1-1 complex that cannot bind TopBP1 has a normal capacity to promote elevated accumulation of TopBP1 on chromatin. Taken together, we propose the following mechanism. First, Rad17 loads 9-1-1 onto DNA. Second, TopBP1 accumulates on chromatin in a manner that depends on both Rad17 and 9-1-1. Finally, 9-1-1 and TopBP1 dock in a Rad17-dependent manner prior to activation of Chk1.

217. *Xenopus* DNA2 is a helicase/nuclease that is found in complexes with replication proteins And-1/Ctf4 and Mcm10 and DSB response proteins Nbs1 and ATM

Karen E. Wawrousek, Barbara K. Fortini, Piotr Polaczek, Lu Chen, Qingquan Liu, William G. Dunphy, Judith L. Campbell

We have used the *Xenopus laevis* egg extract system to study the roles of vertebrate Dna2 in DNA replication and double-strand-break (DSB) repair. We first establish that *Xenopus* Dna2 is a helicase, as well as a nuclease. We further show that Dna2 is a nuclear protein that is actively recruited to DNA only after replication origin licensing. Dna2 co-localizes in foci with RPA and is found in a complex with replication fork components And-1 and Mcm10. Dna2 interacts with the DSB repair and checkpoint proteins Nbs1 and ATM. We also determine the order of arrival of ATM, MRN, Dna2, TopBP1 and RPA to duplex DNA ends and show that it is the same both in S-phase and M-phase extracts. Interestingly, Dna2 can bind to DNA ends independently of MRN, but efficient nucleolytic resection, as measured by RPA recruitment, requires both MRN and Dna2. The nuclease activity of Mre11 is required, since its inhibition delays both full Dna2 recruitment and resection. Dna2 depletion inhibits but does not block resection, and Chk1 and Chk2 induction occurs in the absence of Dna2.

218. The Drf1-dependent kinase interacts with Claspin through a conserved protein motif

Daniel A. Gold, William G. Dunphy

The Dbf4/Drf1-dependent kinase (DDK) is required for the initiation of DNA replication in eukaryotes. Another protein, Claspin, mediates the activation of a cellular checkpoint response to stalled replication forks and is also a regulator of replication. In this study, we found that DDK phosphorylates Claspin *in vitro* and forms a nuclear complex containing Cdc7, Drf1, and Claspin in *Xenopus* egg extracts. In addition, purified Claspin and DDK are capable of a direct *in vitro* interaction. We identified a conserved binding site on Claspin required for its interaction with DDK. This site corresponds to the first of two sequence repeats in the Chk1-binding domain (CKBD) of Claspin. Furthermore, we have established that two amino acids in this motif, D861 and Q866, are essential for the interaction between Claspin and DDK. We found that mutant forms of Claspin incapable of interacting with DDK are still able to associate with and activate Chk1 in response to DNA replication blockages. However, Claspin-depleted egg extracts that have been reconstituted with these mutants of Claspin undergo DNA replication more slowly. These findings suggest that the interaction of DDK with Claspin mediates a checkpoint-independent function of Claspin related to DNA replication.

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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. While this is of course still just a dream, we are developing electron-cryomicroscopy-based technologies to do this for at least the largest structures, hoping to show both how individual proteins work together as large "machines" and how those machines are organized into "assembly lines" within living cells.

The principle technique we're developing and using is electron cryotomography (ECT). Briefly, purified proteins, viruses, or even cell cultures are spread into thin films across EM grids and plunge-frozen in liquid ethane. Quick-freezing causes the water to form vitreous ice around the proteins and other macromolecules, preserving their native structure but solidifying the sample so it can withstand the high vacuum within an electron microscope. Projection images are then recorded through the sample as the sample is tilted incrementally along one or two axes. The microscope we use is one of only a few like it in the world: a 300 kV, helium-cooled, energy-filtered, dual-axis tilting, FEG cryo-TEM with a lens-coupled 4k x 4k CCD. Three-dimensional reconstructions, or "tomograms," are then calculated from the images. In this way we can produce 3-D structures of heterogeneous proteins, viruses, and even whole bacterial cells in near-native states to "molecular" (~4-7 nm) resolution.

The first cells we've begun imaging are small bacteria. Now that over a thousand bacterial genomes have been sequenced, a variety of "omic" technologies are being used to document which genes are transcribed and when, which macromolecules are synthesized and how many of each type are present in the cell, and how they interact in pathways to mediate metabolism and regulate gene expression. Despite this encouraging progress, our persistent ignorance about many of the fundamental physical and mechanical processes that occur in a bacterial life cycle is sobering. We still don’t know, for instance, how bacteria generate and maintain their characteristic shapes, establish polarity, organize their genomes, segregate their chromosomes, divide, and in some cases move. Thus in some sense the "omics" technologies are giving us lists of parts and reactions, but bacterial cells are not merely bags of enzymes. Structural and mechanical details are also needed. This is where ECT is poised to make an important contribution.

In recent years, we have used ECT to show by direct visualization that bacteria do indeed have an elaborate cytoskeleton. We have documented structural details of different cell motility mechanisms, chemoreception apparati, flagellar motors, and metabolic microcompartments. We continue to work on all these subjects and hope also to begin shedding light on the structure and management of the nucleoid and cell wall. In addition, we are also imaging the smallest known eukaryote, *Ostreococcus tauri*.

We have also worked to apply the power of ECT to the structure and maturation of the human immunodeficiency virus type 1 (HIV-1). HIV-1 is an interesting structural story: following its discovery in the mid-1980's, thousands (!) of different structures of its 15 different proteins and pieces of its RNA genome have been solved. Nevertheless we still don't know just how these proteins fit together to form intact, infectious virions, or how their organization changes during assembly, maturation, and infection. The main technical obstacle is that while all HIV-1 virions have the same basic features, each virion is unique in its details. Techniques like X-ray crystallography or NMR spectroscopy, which require a large number of identical objects, have not therefore, been able to reveal "supramolecular" details. So far, we have imaged HIV-1 in its immature and mature states, and are now analyzing these at higher resolution and endeavoring to image HIV-1 structures in living host cells, as well as host factors involved in the HIV-1 life cycle.

Technologically, we are working on optimizing sample preservation, recording better images through improved instrumentation, obtaining more images through automation, and extracting as much biological insight as possible from the images through more sophisticated image processing. For more information, see http://www.jensenlab.caltech.edu.

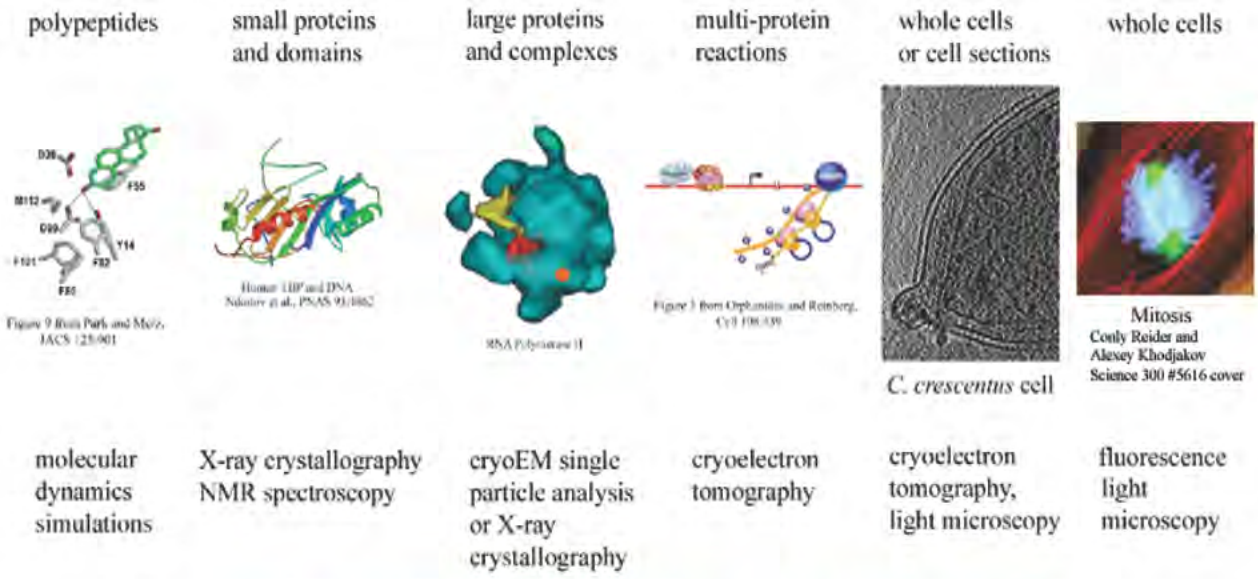

Figure 1. Electron cryotomography completes the "structural biology continuum."

219. Universal architecture of bacterial chemoreceptor arrays

Ariane Briegel[a,b], Davi R. Ortega[c,d], Elitza Tocheva[a], Kristin Wuichet[d], Zhuo Li[a,b], Songye Chen[a], Axel Muller[e], Christina Iancu[a], Gavin Murphy[a], Megan Dobro[a], Igor Zhulin[d,f], Grant J. Jensen[a,b]

Chemoreceptors are key components of the high-performance signal transduction system that controls bacterial chemotaxis. Chemoreceptors are typically localized in a cluster at the cell pole, where interactions among the receptors in the cluster are thought to contribute to the high sensitivity, wide dynamic range, and precise adaptation of the signaling system. Previous structural and genomic studies have produced conflicting models, however, for the arrangement of the chemoreceptors in the clusters. Using whole-cell electron cryo-tomography, here we show that chemoreceptors of different classes and in many different species representing several major bacterial phyla are all arranged into a highly conserved, 12-nm hexagonal array consistent with the proposed "trimer of dimers" organization. The various observed lengths of the receptors confirm current models for the methylation, flexible bundle, signaling, and linker sub-domains *in vivo*. Our results suggest that the basic mechanism and function of receptor clustering is universal among bacterial species and was thus, conserved during evolution.

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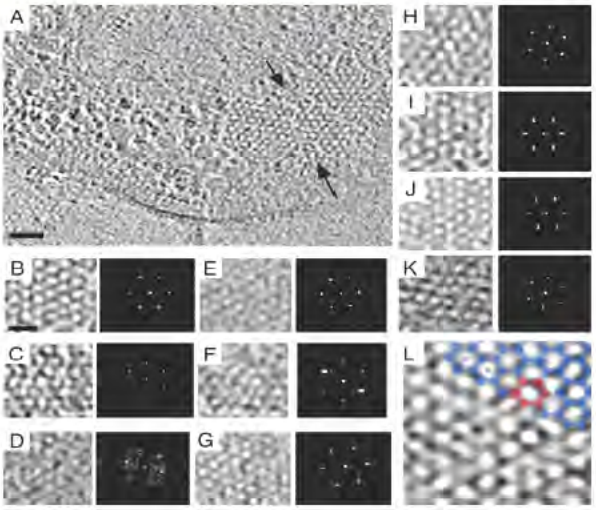

Figure 4. Universally conserved 12-nm hexagonal arrangement of receptor. (*A*) "Top" view of a chemoreceptor array (black arrows) in *T. maritima* (signaling domain class 44H). (Scale bar: 50 nm.) (*B-K*). Top views (*Left*) and power spectra (*Right*) of receptor arrays all reveal the same ≈12-nm hexagonal lattice. *B*, *T. maritima*; *C*, *A. longum*; *D*, *C. jejuni*; *E*, *H. hepaticus*; *F*, *M. magneticum*; *G*, *H. neapolitanus*; *H*, *R. sphaeroides*; *I*, *E. coli*; *J*, *V. cholerae*; *K*, *T. primitia*. (Scale bars: 25 nm; power spectra enlarged.) (*L*) Trimer of dimers (blue) fit into the vertices of the hexagonal lattice in a chemoreceptor array (*V. cholerae*). Six trimers of dimers (red) enclose one hexagon. The spacing from the center of one hexagon to the center of an adjacent one is consistently 12 nm (blue asterisks).

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220. Nanogold as a specific marker for electron cryotomography

Yongning He, Grant J. Jensen, Pamela J. Bjorkman

While electron cryotomography (ECT) provides "molecular" resolution, three-dimensional images of unique biological specimens, sample crowdedness, and/or resolution limitations can make it difficult to identify specific macromolecular components. Here we used a 1.4 nm Nanogold® cluster specifically attached to the Fc fragment of IgG to monitor its interaction with the neonatal Fc receptor (FcRn), a membrane-bound receptor that transports IgG across cells in acidic intracellular vesicles. ECT was used to image complexes formed by Nanogold-labeled Fc bound to FcRn attached to the outer surface of synthetic liposomes. In the resulting three-dimensional reconstructions, 1.4 nm Nanogold particles were distributed predominantly along the interfaces where 2:1 FcRn-Fc complexes bridged adjacent lipid bilayers. These results demonstrate that the 1.4 nm Nanogold cluster is visible in tomograms of typically thick samples (~250 nm) recorded with defocuses appropriate for large macromolecules and is thus, an effective marker.

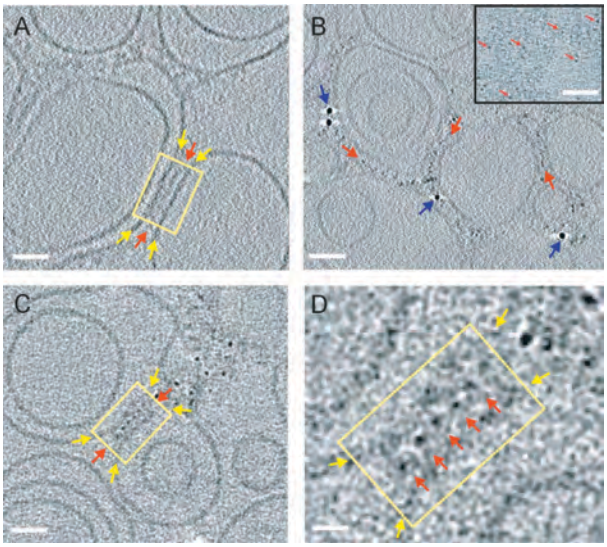

Figure 3. Tomographic slices (6.8 nm each) of FcRn-liposome plus Fc. Similar results were observed in 23 independent tomograms. **A:** Interface (yellow rectangle) formed between two adjacent liposomes bridged by unlabeled Fc showing continuous density (red arrows) between the adjacent membrane bilayers (yellow arrows). Scale bar = 45 nm. **B:** Overview of a field of FcRn-liposomes demonstrating that densities corresponding to 1.4 nm Nanogold-Fc (red arrows) are distributed exclusively at the interfaces between adjacent liposomes. Positions of 10 nm colloidal gold fiducial markers are indicated with blue arrows. Inset: 1.4 nm Nanogold-Fc in the absence of FcRn-liposomes (6.8 nm tomographic slice derived from a region of the grid over a hole). Nanogold clusters were identified as dark densities, each ~2 nm in diameter (2–3 pixel, 0.68 nm/pixel), corresponding to the diameter of a single cluster plus the maleimido protecting groups. Scale bar = 60 nm. **C:** Interface (yellow rectangle) formed between two adjacent liposomes bridged by Nanogold-labeled Fc showing a row of gold clusters (red arrows) between the adjacent membrane bilayers (yellow arrows). Scale bar = 45 nm. **D:** Enlarged view of panel **C** demonstrating that the positions of individual 2:1 FcRn-Fc complexes can be identified by the gold cluster densities (red arrows). Scale bar = 10 nm.

Reference

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221. Organization, structure, and assembly of α-carboxysomes determined by electron cryotomography of intact cells

C.V. Iancu, D.M. Morris, Z. Dou, S. Heinhorst, G.C. Cannon, G.J. Jensen

Carboxysomes are polyhedral inclusion bodies that play a key role in autotrophic metabolism in many bacteria. Using electron cryotomography, we examined carboxysomes in their native states within intact cells of three chemolithoautotrophic bacteria. We found that carboxysomes generally cluster into distinct groups within the cytoplasm, often in the immediate vicinity of polyphosphate granules, and a regular lattice of density frequently connects granules to nearby carboxysomes. Small granular bodies were also seen within carboxysomes. These observations suggest a functional relationship between carboxysomes and polyphosphate granules. Carboxysomes exhibited greater size, shape, and compositional variability in cells than in purified preparations. Finally, we observed carboxysomes in various stages of assembly, as well as filamentous structures that we attribute to misassembled shell protein. Surprisingly, no more than one partial carboxysome was ever observed per cell. Based on these observations, we propose a model for carboxysome assembly in which the shell and the internal RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) lattice form simultaneously, likely guided by specific interactions between shell proteins and RuBisCOs.

Reference

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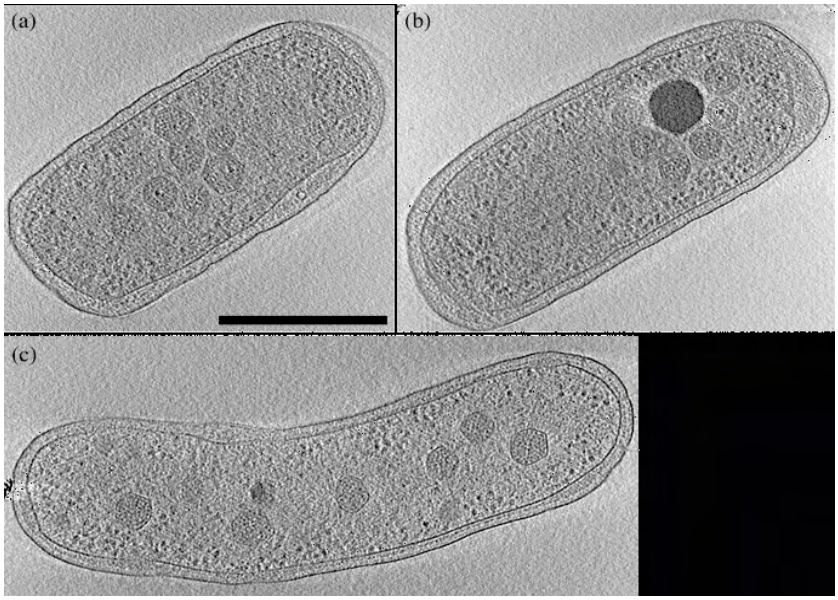

Figure 2. Organization of carboxysomes in *H. neapolitanus* cells. (a) Group of carboxysomes clustered together near the center of a cell. (b) Carboxysomes packed around an electron-dense granule. (c) Carboxysomes dispersed throughout the cell. The scale bar represents 500 nm.

222. Bactofilins, a ubiquitous class of cytoskeletal proteins mediating polar localization of a cell wall synthase in *Caulobacter*

Juliane Kühn[1,2], Ariane Briegel[3,4], Erhard Mörschel[5], Jörg Kahnt[6], Katja Leser[1,2], Stephanie Wick[1,2], Grant J. Jensen[3,4], Martin Thanbichler[1,2]

The cytoskeleton has a key function in the temporal and spatial organization of both prokaryotic and eukaryotic cells. Here, we report the identification of a new class of polymer-forming proteins, termed bactofilins, which are widely conserved among bacteria. In *Caulobacter crescentus*, two bactofilin paralogues cooperate to form a sheet-like structure lining the cytoplasmic membrane in proximity of the stalked cell pole. These assemblies mediate polar localization of a peptidoglycan synthase involved in stalk morphogenesis thus, complementing the function of the actin-like cytoskeleton and the cell division machinery in the regulation of cell wall biogenesis. In other bacteria, bactofilins can establish rod-shaped filaments or associate with the cell division apparatus, indicating considerable structural and functional flexibility. Bactofilins polymerize spontaneously in the absence of additional cofactors *in vitro*, forming stable ribbon- or rod-like filament bundles. Our results suggest that these structures have evolved as an alternative to intermediate filaments, serving as versatile molecular scaffolds in a variety of cellular pathways.

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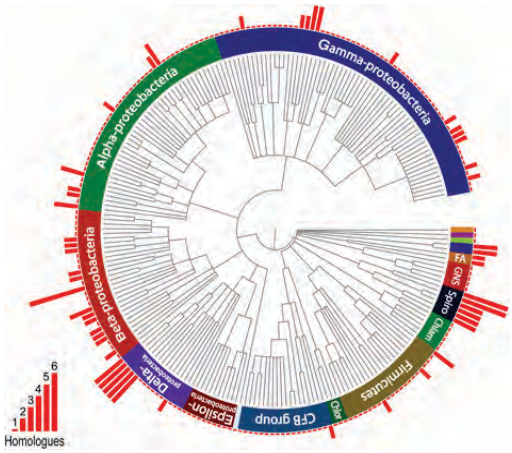

Figure 2. Conservation of bactofilin among bacteria. The PFAM database (Finn *et al.*, 2008) was used to search for bacterial species possessing DUF583-containing proteins. Where sequence information was available for more than one strain per species, only a single strain was chosen for further analysis. After retrieving the corresponding taxonomy IDs from the National Center for Biotechnology Information (NCBI) website, a phylogenetic tree of the species identified was created using the iTOL server (Letunic and Bork, 2007). For each species shown, the number of bactofilin homologues encoded in the genome is indicated by a red bar: Chlor, green sulphur bacteria; Chlam, chlamydias; Spiro, spirochaetes; GNS, green non-sulphur bacteria; FA, fibrobacteres-acidobacteria group.

Publication

Kuhn, J. *et al.* (2010) Bactofilins, a ubiquitous class of cytoskeletal proteins mediating polar localization of a cell wall synthase in *Caulobacter*. *EMBO J.* **29**(2):327-339.

223. CTP synthase forms cytoskeletal filaments that regulate cell shape in *Caulobacter crescentus*

M. Ingerson-Mahar, A. Briegel, J. Werner, G.J. Jensen, Z. Gitai

Filament-forming cytoskeletal proteins are key organizers of all cells. Bacterial homologs of the major eukaryotic cytoskeletal families have now been discovered, but studies suggest that yet more cytoskeletal proteins remain to be identified. Here we demonstrate that the metabolic enzyme CTP Synthase (CtpS) forms filaments in *Caulobacter crescentus*. These filaments are bifunctional and regulate *Caulobacter* curvature independently of CtpS catalytic activity. The morphogenic role of CtpS requires its functional interaction with the intermediate filament crescentin. Interestingly, the *E. coli* CtpS homolog also forms filaments both *in vivo* and *in vitro*, suggesting that CtpS polymerization may be widely conserved. *E. coli* CtpS can replace the enzymatic and morphogenic functions of *Caulobacter* CtpS, indicating that *Caulobacter* has adapted a conserved filament-forming protein for a secondary role. These results implicate CtpS as a novel bifunctional member of the bacterial cytoskeleton and suggest that localization and polymerization may be important properties of metabolic enzymes.

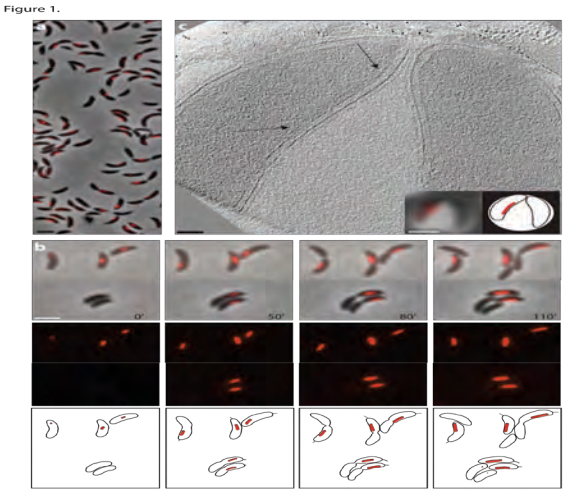

Figure 1. mCherry-CtpS is dynamic and co-localizes with linear filamentous structures along the inner curvature of *Caulobacter* cells. (a) mCherry-CtpS localization in asynchronous *Caulobacter* cells. *mCherry-ctpS* expressing cells (ZG153) were induced for two hours with xylose and imaged. A merged phase and fluorescence image is shown. Scale bar represents 2 μm. (b) Co-localization of mCherry-CtpS and filamentous structures in *Caulobacter*. *mCherry-ctpS* expressing cells (ZG153) were fixed on EM grids and imaged first by fluorescence light microscopy and then by ECT. Shown is an ECT slice, phase/fluorescence overlay (left inset), and cartoon depiction (right inset) of the same cells. These cells correspond to cells #15 from the field shown in Figure S2. Arrows point to the ends of the filaments in the ECT image, which correspond to the positions of the mCherry-CtpS structure shown in the inset. Scale bars represent 100 nm for EM and 1 μm for the inset LM images. (c) mCherry-CtpS localization in synchronized *Caulobacter* cells. *mCherry-ctpS* expressing cells (ZG153) were induced for two hours with xylose, synchronized, and imaged at 10-minute intervals in the presence of xylose. Merged phase and fluorescence (top), fluorescence (middle), and cartoon depictions (bottom) are shown for representative cells at 0, 50, 80, and 110 minutes from the beginning of the timelapse. Scale bar represents 2 μm.

Publication

Ingerson-Mahar, M. *et al.* (2010) CTP synthase forms cytoskeletal filaments that regulate cell shape in *Caulobacter crescentus*. *Nature Cell Biology*. In press.

224. Mutations in the lipopolysaccharide biosynthesis pathway interfere with crescentin-mediated cell curvature in *Caulobacter crescentus*

M.T. Cabeen, M.A. Murolo, A. Briegel, N. Ausmees, G.J. Jensen, C. Jacobs-Wagner

Bacterial cell morphogenesis requires coordination among multiple cellular systems, including the bacterial cytoskeleton and the cell wall. In the vibrioid bacterium *Caulobacter crescentus*, the intermediate filament-like protein crescentin forms a cell envelope-associated cytoskeletal structure that controls cellwall growth to generate cell curvature. We undertook a genetic screen to find other cellular components important for cell curvature. Here we report that deletion of a gene (*wbqL*) involved in the lipopolysaccharide (LPS) biosynthesis pathway abolishes cell curvature. Loss of WbqL function leads to the accumulation of an aberrant O-polysaccharide species and to the release of the S layer in the culture medium. Epistasis and microscopy experiments show that neither S-layer nor O-polysaccharide production is required for curved cell morphology *per se*, but that production of the altered O-polysaccharide species abolishes cell curvature by apparently interfering with the ability of the crescentin structure to associate with the cell envelope. Our data suggest that perturbations in a cellular pathway that is itself fully dispensable for cell curvature can cause a disruption of cell morphogenesis, highlighting the delicate harmony among unrelated cellular systems. Using the *wbqL* mutant, we also show that the normal assembly and growth properties of the crescentin structure are independent of its association with the cell envelope. However, this envelope association is important for facilitating the local disruption of the stable crescentin structure at the division site during cytokinesis.

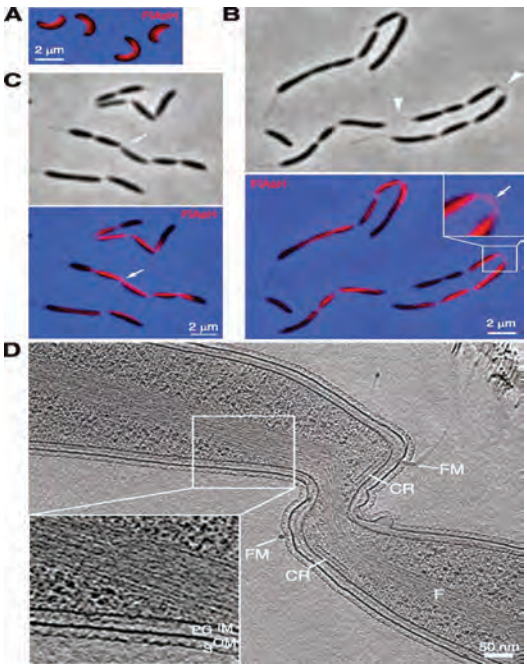

Figure 6. Overproduction of envelope-dissociated crescentin causes cell chaining. (A) Image of fluorescein arsenical helix binder (FlAsH)-labeled crescentin-TC (red) overproduced in a wild-type background (CJW3330; CB15N/pJS14creS-tc cells), laid over phase-contrast micrograph (blue). (B) Micrographs of cells overproducing crescentin-TC in a *wbqL*::pBGENT-KO background (CJW3295; CB15N*wbqL*::pBGENT-KO/pJS14creS-tc). Phase-contrast image is given at top, with an overlay of phase-contrast (blue) with FlAsH-stained crescentin-TC (red) at bottom. Arrowheads indicate visible thin extensions connecting chained cells; inset magnifies the presence of a visible crescentin structure running through one such extension (arrow). (C) Images of FlAsH-stained crescentin N27-TC (red) overproduced (induced for 4 h) as the only source of crescentin in the cell [CJW1537; CB15N *creS*/pMR20P*xyl*creS (N27)-tc], laid over phase-contrast micrograph (blue). Arrow indicates a crescentin N27-TC structure running between chained cells. (D) Section of an electron cryotomogram of two chained CJW914 cells CB15N/pJS14P*xyl*creS (N27)] overproducing crescentin N27, F, filamentous structure; CR, chemoreceptor array; FM, flagellar motor; S, S layer; OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

Publication

Cabeen *et al.* (2010) Mutations in the lipopolysaccharide biosynthesis pathway interfere with crescentin-mediated cell curvature in *Caulobacter crescentus*. *J. Bacteriol.* **192**:3368-3378. http://dx.doi.org/1128/JB.01371-09

225. Cryo-electron tomography of homophilic adhesion mediated by the neural cell adhesion molecule L1

He Yongning, Grant J. Jensen, Pamela J. Bjorkman

The neural cell adhesion molecule L1 participates in homophilic interactions important for axon guidance and neuronal development. The structural details of homophilic adhesion mediated by L1 and other immunoglobulin superfamily members containing an N-terminal horseshoe arrangement of four immunoglobulin-like domains are unknown. Here we used cryo-electron tomography to study liposomes to which intact or truncated forms of the L1 ectodomain were attached. Tomographic reconstructions revealed an adhesion interface with a regular and repeating pattern consistent with interactions between paired horseshoes contributed by L1 proteins from neighboring liposomes. The characteristics of the pattern changed when N-linked carbohydrates were altered by removing sialic acids or converting them from complex to high mannose or oligomannose glycans, which suggests a regulatory role for carbohydrates in L1-mediated homophilic adhesion. Using the results from tomograms and crystal structures of L1-related molecules, we present a structural model for L1-mediated homophilic adhesion that depends on protein-protein, protein-carbohydrate, and carbohydrate-carbohydrate interactions.

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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.

226. Active site-saturation mutagenesis of a designed Kemp elimination enzyme

Alexandria H. Berry, Alex Nisthal*, Stephen L. Mayo*

Enzymes are biological super catalysts. They are highly specific, can function at biological pH and temperature, and accelerate reactions with rate enhancements of 10^5–10^{17}. Enzymes have practical applications as medicinal therapeutics and as catalysts for industrial processes, but enzymes available for such uses have historically been limited to what is found in nature. Therefore, the *de novo* design of enzymes with novel activities has become a major scientific goal. Previous work in the Mayo lab resulted in the successful design of a *de novo* enzyme catalyzing the Kemp elimination: HG-2/S265T. This enzyme was designed using the protein design software PHOENIX and molecular dynamics simulations, and has a k_{cat}/K_m of 430 $M^{-1}s^{-1}$. Although quite an accomplishment, this value is still far lower than the 10^7 $M^{-1}s^{-1}$ values typically found in nature. To further increase the activity of this enzyme and to identify weaknesses in our computational design methods, we performed site-saturation mutagenesis on the active site of HG-2/S265T. The resulting 209 mutants will be expressed and assayed for Kemp elimination activity. Further optimization of our automated mutagenesis procedure is required, but so far all 209 of the mutants have been obtained and are in the process of being catalytically characterized. Optimizing our design parameters so that the predictions made by our computational methods better match that which is observed *in vitro* will improve our model, furthering our long-term goal of designing *de novo* enzymes with k_{cat}/K_m values closer to those found in nature.

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227. Stabilizing TG2 in two conformations with computational multi-state design

Alexandria H. Berry[1], Heidi K. Privett[2], Stephen L. Mayo

Human type II transglutaminase (TG2) is an enzyme that exists in two dramatically different conformational states, each with a unique activity. In the open, extended form, the transglutaminase active site is exposed, allowing TG2 to catalyze formation of an isopeptide bond between the sidechain of a peptide-bound glutamine and a primary amine. Upon GTP binding to a separate GTPase active site, TG2 adopts a compact closed conformation, which obstructs the glutaminase active site and only allows for GTPase activity. TG2 has been linked to Huntington's disease as well as to many other cellular processes, both physiological and pathological. TG2's many roles, as well as it's two conformational states and catalytic activities, have made it difficult to determine exactly how this enzyme functions in disease progression. Also, crystallization of the open form with various small molecule transglutaminase inhibitors has proven difficult due to TG2's preference for the closed state. The goal of this project is to use computational multi-state protein design to engineer TG2 variants locked in either the open or closed conformation. A closed-locked TG2 variant would exhibit only GTPase activity, whereas an open-locked variant would only exhibit transglutaminase activity. These variants will be used in Huntington's disease studies to isolate the effects of each of TG2's activities and to assist in rational drug design.

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228. Using computationally designed libraries to red shift a red fluorescent protein

Roberto A. Chica, Matthew M. Moore, Benjamin D. Allen*, Stephen L. Mayo*

The longer emission wavelengths of red fluorescent proteins (RFPs) make them attractive for whole animal imaging because cells are more transparent to red light. Although several useful RFPs have been developed, the quest for further red-shifted and improved RFPs continues. Using a structure-based rational approach that

combines computational protein design with experimental screening, we were able to obtain mutants of the RFP mCherry that exhibit red-shifted fluorescence emission spectra. We used computational protein design as an in silico pre-screen to generate focused combinatorial libraries of mCherry mutants. The computational procedure helped us identify residues that could fulfill interactions hypothesized to cause red shifts without destabilizing the protein fold. These interactions include stabilization of the excited state through H-bonding to the acylimine oxygen atom, destabilization of the ground state by hydrophobic packing around the charged phenolate, and stabilization of the excited state by a π-stacking interaction. Our methodology allowed us to identify three mCherry mutants (mRojoA, mRojoB, and mRouge) that display emission wavelengths >630 nm, representing red shifts of 20 to 26 nm. Moreover, our approach required the experimental screening of a total of 5000 clones, a number several orders of magnitude smaller than those previously used to achieve comparable red shifts. Additionally, crystal structures of mRojoA and mRouge allowed us to verify fulfillment of the interactions hypothesized to cause red shifts, supporting their contribution to the observed red shifts.

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229. A novel high-resolution protein structure refinement algorithm

Mohsen Chitsaz[*], Stephen L. Mayo

The refinement of low-resolution protein models to generate structures with atomic-level accuracy remains a major challenge in structural biology. Energy-based refinement is mainly dependent on two components: (1) a sufficiently accurate force field, and (2) an efficient algorithm to search the enormous conformational space. Focusing on the latter, we developed a new high-resolution protein structure refinement algorithm called GRID. We compare GRID to Backrub, another algorithm that can predict a significant fraction of the conformational changes that occur with point mutations by incorporating the backbone flexibility associated with correlated backbone side-chain motions [1-2]. We applied GRID and Backrub to 11 high-resolution (~2.8 Å) crystal structures from the Protein Data Bank and measured the energy improvements obtained and the computational effort used by each algorithm. On average, GRID resulted in energy improvements that were ~50% better than those achieved by Backrub while spending about 400-fold less computational resources. Refinement was also performed on a set of decoys generated from the same 11 crystal structures. Decoys were obtained by randomly perturbing all the f and φ angles to result in average backbone RMSDs of ~1 Å. Refinement with GRID consistently decreased these RMSDs (by ~0.4 Å on average), whereas, Backrub caused no improvement (–0.03 Å), with GRID again showing much better performance. We are currently exploring the use of evolutionary algorithms that may further enhance the predictability of GRID and extend its capabilities to allow refinement of lower-resolution structures.

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230. Structural and functional studies of cyanovirin-N dimers

Jennifer R. Keeffe, Sarah K. Gillespie, Stephen L. Mayo

Cyanovirin-N (CV-N), a protein originally isolated from the cyanobacterium *Nostoc ellipsosporum*, binds specifically to high mannose glycosylation present on enveloped virus glycoproteins, preventing viral fusion. We previously reported that covalently linked CV-N dimers (CVN_2s) and trimers (CVN_3s) show enhanced potency for HIV neutralization, with the dimer containing no linker residues (CVN_2L0) showing the most improvement. We obtained high-resolution crystal structures of three CVN_2s and showed that all three are domain-swapped dimers with a high degree of similarity to published wild-type (WT) CV-N dimeric crystal structures. To eliminate the possibility that this domain-swapped form is an artifact of the crystallography process, as it is for WT CV-N, we constructed CVN_2L0 variants with binding site mutations. Each carbohydrate-binding site in the domain-swapped dimer contains residues from both repeats, so we were able to generate mutants in that a complete binding site would be knocked out in the context of either a monomer-like model or a domain-swapped dimer model. We found that the variants with binding site knockouts in the context of the domain-swapped dimer model exhibited decreased HIV neutralization compared to those from the monomer-like model, indicating that the protein is in fact a domain-swapped dimer in solution.

To determine whether CVN_2L0's enhanced potency is due solely to stabilization of the domain-swapped form or is also related to the increase in avidity, we created variants with one, two, or three functional binding sites. We found that CVN_2L0's ability to neutralize HIV is proportional to the number of functional binding sites, confirming an important role for avidity in the antiviral activity of CV-N.

231. Computational design and characterization of a hyperthermostable cellulase

Toni M. Lee[*], Stephen L. Mayo

The degradation of lignocellulose into monomeric glucose units constitutes an essential step in the efficient synthesis of ethanol-based biofuels. Lignocellulose exists as a crystalline lattice of polymeric glucose chains wrapped in highly stable lignin. Upon disruption of the lignin shell and crystalline lattice, cellulases cooperatively catalyze the hydrolysis of cellulose polymers into

monomers. Harsh pretreatment conditions used to expose free cellulose polymers, however, can prematurely denature any enzymes present. As such, one strategy for improving the efficiency of cellulose ethanol production involves the use of cellulases capable of withstanding the high temperature or low pH environment introduced during pretreatment. Computational protein design provides a means of producing such cellulases. To this end, we plan to utilize our design methods to enhance the ability of a highly utilized industrial cellulase, the catalytic domain of Cel5A (EGII) from *Trichoderma reesei*, to withstand high temperatures.

Computational protein engineering requires the pre-existence of a starting structure or scaffold. To this end, we have focused our efforts on obtaining a crystal structure of *T. reesei* Cel5A, a protein for which no high-resolution structural data currently exists. A protocol for soluble expression of this fungal protein in *Escherichia coli* has been developed. Although preliminary crystals have been obtained, they exhibit plate-like growth, hindering the collection of a full diffraction data set. Additionally, the protein precipitates from solution at the high concentrations required for crystallization studies. We are currently in the process of performing site-directed mutagenesis to prevent aggregation through disulfide bonding and increase the probability of growing high-quality crystals. Upon obtaining a *T. reesei* Cel5A crystal structure, computational design will commence to improve thermostability while preserving or enhancing enzymatic function.

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232. *In vitro* studies on cyanovirin-N binding to carbohydrates on viral glycoproteins

Irene Maier, Alexandria H. Berry[*], Jennifer R. Keeffe, Stephen L. Mayo

Transmembrane-linked glycoproteins on the surface of enveloped viruses such as influenza, Ebola, and human immunodeficiency virus (HIV) initiate viral attachment and fusion to host membranes. These glycoproteins carry the epitopic structures recognized by neutralizing antibodies and are thus, the primary targets for the design of anti-viral vaccines and viral entry inhibitors. Their glycosylation also makes them an ideal target for carbohydrate-binding proteins such as cyanovirin-N (CV-N), a cyanobacterial lectin known for its broad neutralization ability [1]. CV-N's anti-viral activity has been attributed to two distinct carbohydrate-binding sites that selectively bind N-linked high-mannose oligosaccharides: one with low affinity, and one with high affinity.

We are developing an *in vitro* immunoassay that uses CV-N to monitor protein-carbohydrate binding on viral envelope glycoproteins. The final methodology should provide a simple and inexpensive way to pre-screen protein variants for their ability to interact with viral carbohydrates and thus, prevent viral membrane fusion and infection. Once the assay has been validated, studies will be aimed at enhancing this specific protein-carbohydrate interaction with computationally designed variants of CV-N. Thus far, we have used surface plasmon resonance and ELISA assays to measure the binding affinity of CV-N to several viral glycoproteins, including gp120 (HIV), hemagglutinin (influenza), and Ebola GP1,2 (Ebola). The glycoproteins were made recombinantly and expressed in either insect or mammalian cells.

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233. Experimental system to study mechanistic theories of backbone cyclization

Matthew M. Moore[*], Stephen L. Mayo

Peptide backbone cyclization is a rare and quite versatile post-translational modification among proteins. Backbone cyclizations are known to occur in green fluorescent protein (GFP) and histidine ammonia-lyase, where their products are used for bioluminescence and chemical reactivity, respectively. This chemical modification is unique for GFP in that it occurs autocatalytically and thus, no external cofactors or enzymes are needed to obtain fluorescence during heterologous expression. The biosynthesis of a fluorophore in GFP by way of backbone cyclization is therefore completely encoded in the primary structure of this protein. The mechanism of this fluorophore formation has been intensely studied, and the knowledge gained has been successfully applied toward the rational engineering of fluorescent protein variants with novel properties. In the hopes of recapitulating the requirements for fluorophore formation in a new scaffold, we identified a protein containing the same topological fold present in all known fluorescent proteins—an 11-stranded β-barrel with a coaxial helix. This fold is formed by residues 400-623 of the murine nidogen-1 G2 domain. A 6X-His tag was appended to this protein segment to facilitate purification by immobilized metal ion chromatography. So far, we have developed a high-yield (19 mg per 1.0 L culture) expression system in *Escherichia coli* and have characterized the recombinant protein by circular dichroism, liquid chromatography-mass spectrometry, surface plasmon resonance, and X-ray crystallography. This work confirmed that the protein has the correct tertiary structure (as seen in the 1.90 Å crystal structure), and that it binds to collagen-IV and laminin with affinities consistent with those reported in the literature. The goal of this project is to use the recombinant protein as a "blank-slate scaffold" to test a variety of mechanistic theories on the causes of autocatalytic backbone cyclization. Computational protein design software developed in-house will be used to guide the selection of mutations that may shed light on this process.

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234. Library designs to expand calmodulin-binding diversity

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Calmodulin (CaM) is a small Ca^{2+}-binding protein that binds to and regulates a number of different protein targets. Its ability to tightly bind to a diverse set of small peptides makes it an ideal system for searching for new binding targets. Hundreds of sequences are already known to bind CaM, and analysis of the interactions involved has led to hypotheses regarding the basic elements required for binding [1]. Several high-resolution CaM-peptide complexes have been solved by solution NMR and X-ray crystallography. Further, the ORBIT computational design software has been successfully used to generate CaM variants with increased specificity toward a particular target [2]. Using this abundant knowledge of CaM binding affinity and specificity, we intend to design libraries of CaM variants to bind novel peptides with high affinity. The designs will be performed using the PHOENIX computational design software. We will also utilize molecular dynamics to further test the designs. We focused on viral proteins as our novel targets in hopes that the variants obtained could eventually be developed for therapeutic use. A BLAST search of the viral genome database was performed to find novel peptide sequences that contain a CaM-binding consensus sequence. Eight sequences were found that satisfy this criteria, and X-ray crystallographic structures are available for two of them. To experimentally validate the CaM libraries, a high-throughput assay was developed to determine CaM-peptide binding. The assay is based on fluorescence resonance energy transfer (FRET), a state-of-the-art method to characterize interactions between molecules. Color variants of green fluorescent protein (GFP) can be attached to a host protein (CaM) and a target peptide. FRET can be used to determine protein-protein interactions in cell lysates thus, allowing the efficient identification of CaM-binding peptides. Using a 96-well plate-based assay, several 100-member libraries designed to bind viral peptides are being tested experimentally.

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235. Protein stability database construction by automated site-directed mutagenesis

Alex Nisthal[*]*, Stephen L. Mayo*

The development of scoring functions for predicting protein stabilities requires large amounts of high quality data. All general-purpose stability prediction software used today was trained on the Protherm database, an aggregate dataset of all stability data reported in the literature. Although extremely useful, this database suffers from the following limitations: (1) data collected are from a wide-ranging set of experimental assays and conditions; (2) only positive measurements are reported, ignoring insoluble/unfolded sequences; and (3) a large percentage of point mutations are from a single protein, T4 lysozyme. As such, only ~2000 independent measurements from the database are commonly used to train prediction software. To address these concerns, we initiated a large-scale project aimed at facilitating the construction of every single mutant of any particular protein domain. Appropriate high-throughput automation technology was acquired and an experimental pipeline for the ordering, generation, sequence verification, expression, purification, and assay of single-site protein mutants was developed. The first domain to be processed was protein G, a 56-residue beta-grasp (ubiquitin-like) fold, which entailed the construction of ~1000 single-site variants. This dataset, housed internally in a mySQL database, is already significant as it contains self-consistent and identically measured data on both folded and unfolded protein sequences. It is anticipated that data will be periodically added for domains composed of ~100 residues, featuring vastly different folds, and exhibiting enzymatic activity. In depth analysis and scoring function optimization with the current dataset is already underway. The current database will be made available to the public in the near future.

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236. Designing protein domains for use in synthetic circuits

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Synthetic biology aims to take advantage of biological processes to engineer systems and devices with novel and useful functions. Since it is still in its infancy, much work is needed in order to build a framework to facilitate design using biological parts. This project aims to contribute to this framework and expand the synthetic biology toolkit. Our goal is to leverage the advances in computational protein design to engineer domains that can be used in synthetic circuits. Specifically, we intend to design domains that change conformation in the presence of a stimulus. By inserting these domains into different proteins, we hope to obtain a general method for stimulus-dependent modulation of protein activity. Currently, we are looking at phosphorylation of the domain as the mechanism for inducing the conformational change. Ideally, the domain will contain a region that is a target for an "orthogonal" inducible kinase. Upon input of a stimulus, the kinase will phosphorylate the target region, causing a conformational change that will modulate protein activity. Phosphorylation events are widely used in natural systems to control the dynamics of gene networks. The two negative charges that phosphorylation adds to the protein cause changes in the electrostatics of the protein, which can affect its binding state or activity. Phosphorylation also acts on many different timescales (ranging from less than a second to hours), which are

tunable by the kinetics of the dephosphorylation reaction. Successful design of this device will provide a new tool in the control of synthetic circuits, allowing for fast, reversible control of protein activity.

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237. A molecular dynamics approach for prescreening computational enzyme designs

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Computational enzyme design is a very promising area of research. However, current computational design tools suffer from a serious limitation: they cannot reliably predict active sequences and thus, produce a large number of false positives. Consequently, investigators have had to rely on the experimental screening of hundreds of designs to find a few active ones. In addition, although both protein dynamics and explicit solvation are important aspects of enzyme function and are therefore critical to predicting enzymatic activity, protein design methodology rarely takes these factors into account. Molecular dynamics (MD) simulations, on the other hand, can model both. We therefore, decided to develop a MD protocol that would provide a reliable and computationally feasible way to pre-screen enzyme designs. Preliminary results showed an outstanding correlation between experimental data and MD predictions. We are currently testing the generality of the methodology on a larger and more diverse set of enzymes for which experimental data already exist. The method is highly accurate and reproducible, and exhibits promising computational performance.

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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, Israel) and by my laboratory, then at MIT. These discoveries revealed three sets of previously unknown facts:

1. ATP-dependent protein degradation involves a new protein modification, the conjugation of ubiquitin, a 76-residue protein, to other proteins. This conjugation is mediated by specific enzymes, termed E1, E2 and E3.

2. Ubiquitin is a 'secondary' degradation signal in that ubiquitin is conjugated to proteins that contain primary degradation signals (degrons). These signals underlie the specificity of the ubiquitin system. They include degrons that give rise to the N-end rule of protein degradation.

3. 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 shown by us to be required for the bulk of protein degradation *in vivo*, for cell viability, and also, specifically, for the cell cycle, DNA repair, protein synthesis, transcriptional regulation, and stress responses. That was done, in part, by discovering that the previously known but biochemically undefined DNA repair regulator RAD6 and the cell cycle regulator CDC34 were ubiquitin-conjugating (E2) enzymes, the first such enzymes with specific physiological functions. We also cloned the first ubiquitin genes (discovering their different functions), the first specific E3 ubiquitin ligase (UBR1), the first deubiquitylating enzymes (DUBs), and identified the first physiological substrate of the ubiquitin system, the MATalpha2 transcriptional repressor. In addition, we discovered that ubiquitin-mediated proteolysis requires substrate-linked polyubiquitin chains with specific isopeptide bonds between adjacent ubiquitin moieties. The ubiquitin system was also shown to possess the fundamental property of subunit selectivity, i.e., the ability to destroy, selectively, a specific subunit of an oligomeric protein, leaving the rest of the protein intact and thereby making possible protein remodeling. This critical ability underlies the cell cycle (selective replacements of cyclin subunits in oligomeric cell cycle kinases), the activation of transcription factors such as, for example, NF-kappaB, and a legion of other biological processes.

The Hershko laboratory produced the first of these fundamental advances (item 1), and my laboratory produced the other two (items 2 and 3). Our function-based studies in the 1980s yielded the overall discovery of the *physiological regulation by intracellular protein degradation*. The complementary 'chemical' and 'biological' insights by the Hershko's and my laboratories led to a massive expansion of the ubiquitin field in the 1990s. It became one of the largest arenas in biomedical science, the point of convergence of many disparate disciplines. Because perturbations of the cell cycle, DNA repair and stress response pathways are hallmarks of malignant transformation, our 1987-88 discoveries with CDC34 (ubiquitin-conjugating enzyme required for the cell cycle), RAD6 (ubiquitin-conjugating enzyme required for DNA repair) and UBI4 (a precursor of ubiquitin whose induction is essential for stress responses) opened up ubiquitin studies in cancer research as well. For accounts of the early history of the Ub field, see Hershko *et al*. (2000); Varshavsky (2006, 2008).

Together, the above insights in the 1980s produced the modern paradigm of cellular physiology, in which regulated protein degradation is of central importance. These advances, in conjunction with later studies by many laboratories, have revealed that the control through regulated protein degradation rivals, and often surpasses in significance the classical regulation through transcription and translation. This altered understanding of the design of biological circuits is of major significance for 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 the ubiquitin system and regulated protein degradation.

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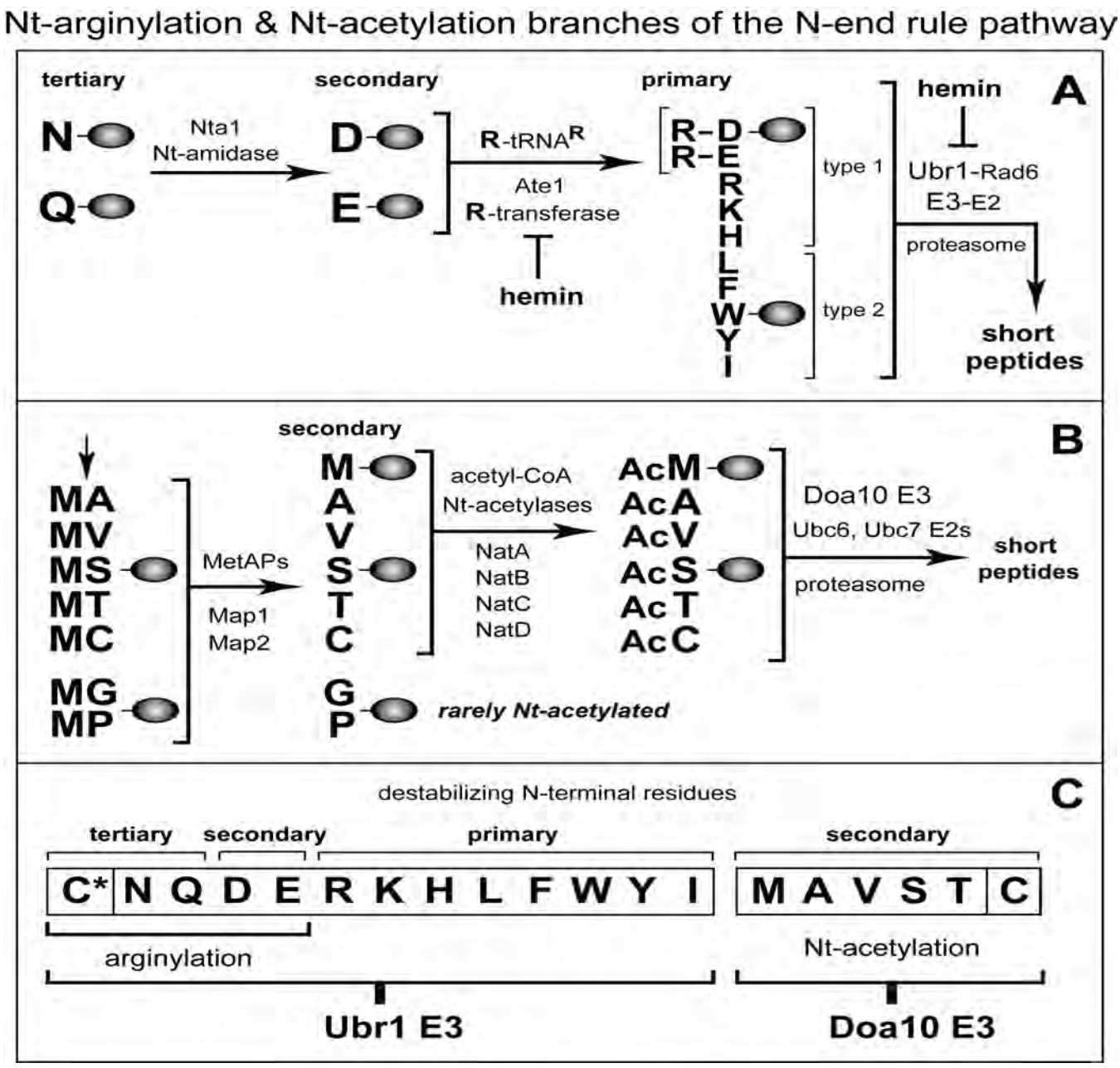

Fig. 1. The N-end rule pathway of the yeast *S. cerevisiae*. It consists of two major branches. The *Arg/N-end rule pathway* (**A**) involves the N-terminal arginylation (Nt-arginylation) of specific substrates and also the targeting of unmodified bulky hydrophobic and basic N-terminal residues (including Arg) by E3 N-recognins (Ub ligases that recognize specific N-degrons) (Varshavsky, 2008). The *Ac/N-end rule pathway* (**B**) involves the cotranslational N-terminal acetylation (Nt-acetylation) of nascent proteins whose N-termini bear either Met or small uncharged residues (Ala, Val, Set, Thr or Cys) (Hwang *et al*., 2010a). The latter residues become N-terminal after the cotranslational removal of Met by Met-aminopeptidases. Nt-acetylated proteins are targeted for polyubiquitylation and proteasome-mediated degradation by the Ac/N-end rule pathway (Hwang *et al*., 2010a). Diagrams in panel **A** were simplified by omitting the recently discovered *physical complex* of two different E3/E2 Ub ligases, Ubr1/Rad6 and Ufd4/Ubc4. The Arg/N-end rule pathway is mediated by this complex, rather than by Ubr1/Rad6 alone (Hwang *et al*., 2010b). (**C**) N-terminal residues that are targeted by the Arg/N-end rule and Ac/N-end rule pathways. Both branches can target, through different mechanisms, the N-terminal Cys residue of protein substrates. See the main text for definitions of the 'primary,' 'secondary,' and 'tertiary' destabilizing N-terminal residues.

Ubiquitin (Ub)-mediated processive 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 Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a Cys residue

of E1. In the second step, activated Ub is transesterified to a Cys residue of an Ub-conjugating (E2) enzyme. Thereafter a complex of E2 and another enzyme, E3, conjugates Ub to a Lys residue of a substrate. 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. Ub has nonproteolytic functions as well.

The N-end rule relates the regulation of the *in vivo* half-life of a protein to the identity of its N-terminal residue (Varshavsky, 2008). N-terminal degradation signals of the N-end rule pathway are called N-degrons. The main determinant of an N-degron is a destabilizing N-terminal residue of a protein. In eukaryotes, the N-end rule pathway is a part of the Ub system. Recognition components of the N-end rule pathway are called N-recognins. In eukaryotes, an N-recognin is an E3 Ub ligase that can target a subset of N-degrons. A complex of E3 N-recognin and its cognate E2 Ub-conjugating enzyme polyubiquitylates N-end rule substrates at their internal Lys residues, thereby, targeting these proteins for processive degradation by the 26S proteasome. The term 'Ub ligase' denotes either an E2-E3 holoenzyme or its E3 component. In eukaryotes, the N-end rule pathway comprises two major branches. One of them is termed the Arg/N-end rule pathway. Until recently (Hwang *et al*., 2010a), this branch was the only known N-end rule pathway in eukaryotes. It involves the N-terminal arginylation (Nt-arginylation) of specific substrates and also the targeting of unmodified bulky hydrophobic and basic N-terminal residues (including Arg) by E3 N-recognins (Fig. 1A). The other branch is termed the Ac/N-end rule pathway (Hwang *et al*., 2010a) (Abstract 244). It involves the cotranslational N-terminal acetylation (Nt-acetylation) of nascent proteins whose N-termini bear either Met or small uncharged residues (Ala, Val, Set, Thr or Cys). The latter residues become N-terminal after the cotranslational removal of Met by Met-aminopeptidases. Nt-acetylated proteins are targeted for polyubiquitylation and proteasome-mediated degradation by the Ac/N-end rule pathway (Fig. 1B, C and Abstract 244).

The N-terminal residues Arg, Lys, His, Leu, Phe, Tyr, Trp, Ile, Asp, Glu, Asn, Gln (and also Cys in organisms that produce nitric oxide (NO)) comprise N-degrons of the Arg/N-rule pathway (Fig. 1A). Among these N-degrons, the unmodified basic (Arg, Lys, His) and bulky hydrophobic (Leu, Phe, Tyr, Trp, Ile) N-terminal residues are recognized directly by cognate E3 N-recognins. Together, the unmodified and directly recognized N-terminal Arg, Lys, His, Leu, Phe, Tyr, Trp and Ile are termed the 'primary' destabilizing residues. In contrast, the N-terminal Asp, Glu, Asn and Gln function as destabilizing residues through their preliminary modifications. One of these modifications is Nt-arginylation, which is mediated by the Ate1 Arg-tRNA-protein transferase, termed R-transferase. N-terminal Arg can be recognized directly by N-recognins of the Arg/N-rule pathway (Fig. 1A). In contrast to N-terminal Asp and Glu, the N-terminal Asn and Gln residues cannot be Nt-arginylated by Ate1. However, the Arg/N-rule pathway contains specific Nt-amidases (Abstracts 241, 242) that convert Asn and Gln to Asp and Glu, respectively, followed by their Nt-arginylation (Fig. 1).

At least four N-recognins, including Ubr1, mediate the Arg/N-end rule pathway in mammals. The known N-recognins share a ~70-residue motif called the UBR box. Mouse Ubr1 and Ubr2 are sequelogous (similar in sequence) 200-kD RING-type E3 Ub ligases that are 47% identical. Several other UBR-containing N-recognins, either confirmed or putative ones, are HECT-type or SCF-type E3 Ub ligases that share the UBR motif with the RING-type Ubr1/Ubr2 but are largely nonsequelogous to them otherwise. (*A note on terminology*: 'sequelog' and 'spalog' denote, respectively, a sequence that is similar, to a specified extent, to another sequence, and a 3D (spatial) structure that is similar, to a specified extent, to another 3D structure (Varshavsky, 2004). Besides their usefulness as separate terms for sequence and spatial similarities, the rigor-conferring advantage of 'sequelog' and 'spalog' is their *evolutionary neutrality*, 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 used to convey understanding about functions and common descent, if this (additional) information is available.

Physiological functions of the N-end rule pathway are strikingly broad. Regulated degradation of specific proteins by the Arg/N-rule pathway mediates the sensing of heme, NO, oxygen and short peptides; the selective elimination of misfolded proteins; the regulation of DNA repair, segregation and condensation; the signaling by transmembrane receptors (through degradation of G-protein regulators such as Rgs4, Rgs5 and Rgs16); the control of peptide import (through degradation of import's repressor); the regulation of apoptosis, meiosis, viral infections, fat metabolism, cell migration, actin filaments, cardiovascular development, spermatogenesis, neurogenesis and memory; the functioning of adult organs, including the brain, muscle, testis and pancreas; and the regulation of leaf and shoot development, leaf senescence and seed germination in plants. The recently discovered Ac/N-rule pathway (Fig. 1B, C) (Hwang *et al*., 2010a) (Abstract 244) is likely to mediate, among other things, protein quality control and degradation of long-lived proteins.

Functional and mechanistic studies of the N-end rule pathway in yeast and mammals are a major theme of our current work.

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238. Ablation of arginylation in the Arg/N-end rule pathway: loss of fat, increased metabolic rate, damaged spermatogenesis, and neurological perturbations

Christopher Brower, Alexander Varshavsky

In the N-end rule pathway of protein degradation, the destabilizing activity of N-terminal Asp, Glu or (oxidized) Cys residues requires their conjugation to Arg, which is recognized directly by pathway's ubiquitin ligases. N-terminal arginylation is mediated by the Ate1 arginyltransferase, whose physiological substrates include the Rgs4, Rgs5 and Rgs16 regulators of G proteins. In this study (Brower & Varshavsky, 2009), we employed the Cre-lox technique to uncover new physiological functions of N-terminal arginylation in adult mice. We showed that postnatal deletion of mouse *Ate1* (its unconditional deletion is embryonic lethal) causes a rapid decrease of body weight and results in early death of ~15% of Ate1-deficient mice. Despite being hyperphagic, the surviving Ate1-deficient mice contain little visceral fat. They also exhibit an increased metabolic rate, ectopic induction of the Ucp1 uncoupling protein in white fat, and are resistant to diet-induced obesity. In addition, Ate1-deficient mice have enlarged brains, an enhanced startle response, are strikingly hyperkinetic, and are prone to seizures and kyphosis. Ate1-deficient males are also infertile, owing to defects in *Ate1*$^{-/-}$ spermatocytes. The remarkably broad range of biological processes that are shown here to be perturbed by the loss of N-terminal arginylation will facilitate the discovery of specific circuits that involve Ate1 and either its known substrates, such as Rgs4, Rgs5 and Rgs16, or those currently unknown.

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239. Two proteolytic pathways regulate DNA repair by co-targeting the Mgt1 alkylguanine transferase

Cheol-Sang Hwang, Anna Shemorry, Alexander Varshavsky

Since the discovery that a key DNA repair protein Rad6 was a Ub-conjugating enzyme (Jentsch *et al*., 1987), there have been great strides in understanding the massive, multi-level involvement of the Ub-proteasome system in the DNA damage response. A major aspect of this response is the repair of damage caused by alkylating agents such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and methyl methane sulfonate (MMS), which produce both mutagenic and cytotoxic lesions in DNA. One functionally severe lesion in double-stranded DNA is O^6-methylguanine (O^6meG), which is demethylated by the O^6-alkylguanine-DNA alkyltransferase (AGT). This protein is called Mgmt (or MGMT) in mammals and Mgt1 in the yeast *S. cerevisiae*. Compounds that produce O^6meG in DNA are common environmental carcinogens. Some of these compounds are also formed as a part of normal cellular metabolism. The repair of O^6meG in DNA is downregulated in many cancers, usually because of lower than normal levels of Mgmt in cancer cells. Consequently, some anticancer drugs are DNA alkylating agents whose targets include O^6 in guanine. An acquired or pre-existing resistance of cancer cells to such drugs often involves an upregulation of Mgmt. AGT proteins remove methyl and other alkyl groups from alkylated O^6 in guanine by transferring an adduct to an active-site Cys residue. The resulting S-alkyl-Cys residue of AGT is not restored back to Cys, so repair proteins of this kind can act only once. In mammals, the alkylated (inactive) Mgmt, and possibly also the unmodified Mgmt are short-lived proteins, degraded by an unknown pathway.

In 2009, we, we discovered that *S. cerevisiae* Mgt1 is co-targeted for degradation, through a degron near its N-terminus, by two ubiquitin-mediated proteolytic systems, the Ubr1/Rad6-dependent N-end end rule pathway and the Ufd4/Ubc4-dependent UFD (ubiquitin fusion degradation) pathway (Hwang *et al*., 2009). The co-targeting of Mgt1 by these pathways is synergistic, in that it increases not only the yield of polyubiquitylated Mgt1, but also the processivity of polyubiquitylation. The N-end rule and UFD pathways co-mediate both the constitutive and MNNG-accelerated degradation of Mgt1. Yeast cells lacking the Ubr1 and Ufd4 ubiquitin ligases were hyperresistant to MNNG but hypersensitive to the toxicity of overexpressed Mgt1. The N-end rule and UFD pathways were the first specific pathways of the Ub system to be discovered (Bachmair *et al*., 1986). Studies of these pathways have been proceeding largely in parallel, until the above discovery of their functional and mechanistic connection. Continuation of this work led to the recent demonstration of a physical complex between Ubr1 and Ufd4 (Hwang *et al*., 2010) (Abstract 246).

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240. The N-end rule pathway controls multiple functions during *Arabidopsis* shoot and leaf development

Emmanuelle Graciet[1], Franziska Walter[1], Diarmuid Ó Maoiléidigh[1], Stephan Pollmann[2], Elliot M. Meyerowitz[3], Alexander Varshavsky[3], Frank Wellmer[1]

In plants, Ub-dependent processes play major and diverse roles, including the regulation of signaling by phytohormones such as auxin, gibberellins and jasmonic acid. In both the yeast *S. cerevisiae* and the mouse an R-transferase is encoded by a single gene, whereas the model plant *Arabidopsis thaliana* contains two closely related R-transferases, AtATE1 (At5g05700) and AtATE2 (At3g11240). Primary destabilizing residues are recognized by E3 Ub ligases of the Arg/N-end rule pathway, called N-recognins. A single N-recognin, Ubr1, is present in *S. cerevisiae*. In contrast, mammalian

genomes encode at least four distinct N-recognins, while in plants, two N-recognins, termed PROTEOLYSIS 1 (PRT1) and PRT6, have been identified in *Arabidopsis*, but other N-recognins are likely to be present, as well.

In the present study, we showed that the *Arabidopsis* R-transferases AtATE1 and AtATE2 regulate various aspects of leaf and shoot development. We also showed that the previously identified N-recognin PROTEOLYSIS6 (PRT6) mediates these R-transferase-dependent activities. We further demonstrated that the arginylation branch of the N-end rule pathway plays a role in repressing the meristem-promoting *BREVIPEDICELLUS* (*BP*) gene in developing leaves. *BP* expression is known to be excluded from *Arabidopsis* leaves by the activities of the ASYMMETRIC LEAVES1 (AS1) transcription factor complex and the phytohormone auxin. Our results suggest that AtATE1 and AtATE2 act redundantly with AS1, but independently of auxin, in the control of leaf development.

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241. Glutamine-specific N-terminal amidase, a component of the Arg/N-end rule pathway

Haiqing Wang[*]*, Konstantin I. Piatkov, Christopher S. Brower, Alexander Varshavsky*

The N-end rule has a hierarchic structure. N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their enzymatic deamidation, to yield the secondary destabilizing N-terminal residues Asp and Glu. Destabilizing activity of N-terminal Asp and Glu requires their conjugation to Arg, one of the primary destabilizing residues, by R-transferase. In *S. cerevisiae*, the deamidation branch of the Arg/N-end rule pathway is mediated by the Nta1 $Nt^{N,Q}$-amidase, which can deamidate either Asn or Gln at the N-termini of polypeptide substrates (Baker and Varshavsky, 1995). In mammals and other multicellular eukaryotes, N-terminal Asn and Gln are deamidated by N-terminal amidases (Nt-amidases) of two kinds. One of them, the previously characterized Ntan1 Nt^{N}-amidase, is specific for N-terminal Asn (Grigoryev *et al*., 1996). In part through analyses of *Ntan1*$^{-/-}$ mice, which could not deamidate N-terminal Asn (Kwon *et al*., 2000), it has been inferred that there also exists a Gln-specific Nt^{Q}-amidase.

In the present work (Wang *et al*., 2009), we detected the activity of Nt^{Q}-amidase, termed Ntaq1, in mouse tissues, purified Ntaq1 from bovine brains, identified its gene, and began studies of this previously undescribed enzyme. The sequence of mouse Ntaq1 (Nt^{Q}-amidase) is highly conserved among animals, plants and some fungi, but is dissimilar to sequences of other amidases, including the N-terminal amidases Ntan1 (Nt^{N}-amidase) and Nta1 ($Nt^{N,Q}$-amidase). A *tungus* mutant in the previously uncharacterized *Drosophila melanogaster Cg8253* gene was found to have defective long-term memory (Dubnau *et al*., 2003). We found that this *Drosophila* gene encodes the counterpart of mouse Ntaq1. In addition, previous proteomic studies identified ~15 putative protein ligands of an uncharacterized human protein encoded by *C8orf32* ((Lim *et al*., 2006). We found that C8orf32 is the human Ntaq1 Nt^{Q}-amidase. Remarkably, 'high-throughput' crystallographic studies of human proteins have recently solved the crystal structure of C8orf32 (Ntaq1). In conjunction with its crystal structure, our site-directed mutagenesis of Ntaq1 indicated that the active site and catalytic mechanism of Nt^{Q}-amidase are similar to those of transglutaminases. Thus, the discovery and characterization of Nt^{Q}-amidase as a component of the N-end rule pathway were 'instantly' complemented by a crystal structure of this enzyme, a set of its putative protein ligands, and evidence for its role in memory processes.

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Wang, H., Piatkov, K.I., Brower, C.S. and Varshavsky, A. (2009) *Mol. Cell* **34**:686-695.

242. Studies of the mouse Nt^{Q}-amidase, Nt^{N}-amidase and R-transferase

Konstantin I. Piatkov, Christopher S. Brower, Alexander Varshavsky

We continue functional and mechanistic analyses of the mouse *Ntaq1*-encoded Nt^{Q}-amidase, *Ntan1*-encoded Nt^{N}-amidase, and *Ate1*-encoded R-transferase. This work includes the previously constructed conditional and nonconditional *Ate1(-/-)* and *Ntan1(-/-)* mouse strains, and also a recently constructed and characterized *Ntaq1(-/-)* mouse strain, which lacks Nt^{Q}-amidase and therefore cannot deamidate N-terminal Gln. Our experiments involve functional (including behavioral) examinations of these mouse mutants and biochemico-genetic approaches to identifying physiological ligands (including substrates) of the above enzymes.

243. Mouse Dfa is a repressor of TATA-box promoters and interacts with the Abt1 activator of basal transcription

Christopher S. Brower, Alexander Varshavsky

Previous studies the mouse Ate1 R-transferase (a component of the Arg/N-end rule pathway) by our laboratory have shown that *Ate1* pre-mRNA is produced from a bidirectional promoter that also expresses, in the opposite direction, a previously uncharacterized gene (Hu *et al*., 2006). In the present work (Brower *et al*., 2010), we began analyzing this gene, termed *Dfa* (divergent from Ate1). Mouse *Dfa* was found to be transcribed from both the bidirectional $P_{Ate1/Dfa}$ promoter and other nearby promoters. The resulting transcripts are alternatively spliced, yielding a complex set of *Dfa* mRNAs that are present largely, though not exclusively, in the testis. A specific *Dfa* mRNA encodes, via its 3'-terminal exon, a 217-residue protein termed Dfa^A. Other *Dfa* mRNAs also contain this exon. Dfa^A is sequelogous (similar in sequence) to a region of the human/mouse HTEX4 protein, whose physiological function is unknown. We produced an affinity-purified antibody to recombinant mouse Dfa^A that detected a 35 kDa protein in the mouse testis and in several cell lines. Experiments in which RNAi was used to down-regulate *Dfa* indicated that the 35 kDa protein was indeed Dfa^A. Furthermore, Dfa^A was present in the interchromatin granule clusters (IGCs), and was also found to bind to the Ggnbp1 gametogenetin-binding protein-1 and to the Abt1 activator of basal transcription that interacts with the TATA-binding protein (TBP). Given these results, RNAi was used to probe the influence of Dfa levels in luciferase reporter assays. We found that Dfa^A acts as a repressor of TATA-box transcriptional promoters.

Publication

Brower, C. S., Veiga, L., Jones, R.H., and Varshavsky, A. (2010) Mouse Dfa is a repressor of TATA-box promoters and interacts with the Abt1 Activator of basal transcription. *J. Biol. Chem.* **285**:17218-17234.

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244. N-terminal acetylation of cellular proteins creates specific degradation signals

Cheol-Sang Hwang, Anna Shemorry, Alexander Varshavsky

We recently discovered that Nα-terminal acetylation (Nt-acetylation) of cellular proteins creates specific degradation signals (degrons), termed AcN-degrons (Hwang *et al*., 2010). These degradation signals are targeted by the novel branch of the N-end rule pathway, termed Ac/N-end rule pathway, (see Fig. 1 and Introduction). AcN-degrons are the largest class of degradation signals in cellular proteins, since e.g., more than 80% of human proteins are cotranslationally (and apparently irreversibly) Nt-acetylated. Thus, remarkably, the majority of eukaryotic proteins acquire a specific degradation signal from the moment of their birth. In S. *cerevisiae* the Ac/N-end rule pathway is mediated by the 151-kD RING-type Doa10 E3 Ub ligase, an integral membrane protein that targets both 'soluble' (nuclear and cytosolic) and transmembrane proteins for polyubiquitylation and proteasome-mediated degradation (Hwang *et al*., 2010). The Doa10 N-recognin is located in the endoplasmic reticulum (ER) membrane and the inner nuclear membrane (INM), which is contiguous with the ER. This E3 was shown to recognize (physically bind to) the Nt-acetylated residues Met, Ala, Val, Ser, Thr, Cys, Gly and Pro. The above Nt-acetylated N-terminal residues are secondary destabilizing residues in the N-end rule, since they must be modified (Nt-acetylated) before they can be recognized and polyubiquitylated by Doa10, in conjunction with the Ubc6/Ubc7 E2s. In contrast to other Nt-acetylatable residues, N-terminal Gly and Pro are rarely Nt-acetylated. Thus, operationally, N-terminal Gly and Pro are often (though not always) inactive as destabilizing residues.

These and related results revealed the main physiological function of Nt-acetylation, producing the largest increase in the scope of the N-end rule pathway since its discovery more than two decades ago. Out of 20 amino acids in the genetic code, at least 18 are now known to function as destabilizing N-terminal residues in the N-end rule pathway (Fig. 1). The Nt-acetylation is largely cotranslational, apparently irreversible, and involves a majority of cellular proteins. What specific functions are subserved by such a massive production of ^{Ac}N-degrons in nascent proteins if many of these proteins are destined for long half-lives? A major role of these degradation signals is likely to involve quality control mechanisms and regulation of protein stoichiometries in a cell. A key feature of such mechanisms would be conditionality of ^{Ac}N-degrons. If a nascent Nt-acetylated protein can fold its N-terminal domain rapidly enough, or if this protein either interacts with a "protective" chaperone such as Hsp90 or becomes assembled into a cognate multisubunit complex, the cotranslationally created ^{Ac}N-degron of this protein may become inaccessible to the Doa10 Ub ligase. Consequently, the degradation of this protein would be decreased or precluded. In contrast, delayed or defective folding of a protein's N-terminal domain (because of oxidative, heat or other stresses, or a conformation-perturbing mutation, or nonstoichiometric levels of cognate protein ligands) would keep an ^{Ac}N-degron exposed (active) and thereby increase the probability of the protein's destruction.

The discovery that Nt-acetylation is a part of the N-end rule pathway (Hwang *et al*., 2010) has also revealed the physiological functions of Nt-acetylases and Met-aminopeptidases. Nt-acetylases produce ^{Ac}N-degrons, while the upstream Met-aminopeptidases, by cleaving off N-terminal Met (Fig. 1B) make possible these degradation signals, all of them except the one mediated by Nt-acetylated Met. Nt-acetylases and Met-aminopeptidases are universally present, extensively characterized and essential enzymes whose physiological roles were largely unknown. These enzymes are now functionally understood components of the N-end rule pathway.

The topologically unique location of N-terminal residues, their massive involvement in proteolysis, and their extensive modifications make N-degrons a particularly striking example of the scope and subtlety of regulated protein degradation.

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245. Further studies of the Ac/N-end rule pathway

Cheol-Sang Hwang, Anna Shemorry, Jang-Hyun Oh, Brandon Wadas, Alexander Varshavsky

The discovery of ^{Ac}N-degrons and the Ac/N-end rule pathway (Hwang *et al*., 2010) (Abstract 244) opened up a number of vistas. Our studies of this new branch of the N-end rule pathway involve its mechanistic dissection and functional characterization, primarily in *S. cerevisiae* and in the mouse. Some of the current projects aim to identify functional roles of ^{Ac}N-degrons in specific cellular proteins, both in yeast and mammals. We also plan to dissect this pathway genetically in mice, in part through construction of mouse strains that lack Teb4, the E3 Ub ligase and a sequelog of Doa10, the E3 that mediates the Ac/N-end rule pathway in *S. cerevisiae*. In addition, we wish to clarify the currently unclear issue of cotranslational versus posttranslational Nt-acetylation of cellular proteins, given the preferential association of Nt-acetylases with ribosomes and the resulting uncertainty about the relative efficacy of posttranslational Nt-acetylation. We also aim to improve both directness and accuracy of degradation assays, in part because a more detailed understanding of ^{Ac}N-degrons will require information about their *in vivo* kinetics that the current assays cannot provide.

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246. The N-end rule pathway is mediated by a complex of the RING-type Ubr1 and HECT-type Ufd4 ubiquitin ligases

Cheol-Sang Hwang, Anna Shemorry, Alexander Varshavsky

Our previous study (Hwang *et al*., 2009) showed that the *S. cerevisiae* Mgt1 DNA repair protein is co-targeted for degradation by the Ubr1/Rad6-mediated Arg/N-end rule pathway and the Ufd4/Ubc4-mediated Ub-fusion degradation (UFD) pathway (Abstract 239). Rad6 and Ubc4/Ubc5 are E2 enzymes that function with the E3s Ubr1 and Ufd4, respectively. Ufd4 is the 168 kDa HECT-type E3 of the UFD pathway. The UFD pathway was discovered in 1986, simultaneously with the N-end rule pathway (Bachmair *et al*., 1986), through analyses of N-terminal Ub fusions in which the Pro residue at the Ub-reporter junction or (in later studies) mutations of the Ub moiety were found to inhibit the cleavage of a fusion by deubiquitylases (DUBs). Such UFD substrates are targeted for degradation through their N-terminal Ub moieties.

We recently discovered that the RING-type Ubr1 E3 and the HECT-type Ufd4 E3 interact, both physically and functionally. Using *in vitro* and *in vivo* approaches, we found that the Ubr1-Ufd4 complex mediates the Arg/N-end rule pathway and a part of the UFD pathway as well. Cooperation, in their physical complex, between Ubr1 and Ufd4 includes their ability to increase the processivity of polyubiquitylation of both Arg/N-end rule and UFD substrates, in comparison to targeting by Ubr1 or Ufd4 alone. Thus, operationally, the complex of Ubr1 and Ufd4 functions as an E3-E4 pair in which the 'assignment' of an E3 or E4 function depends on the substrate and the nature of its degron. We also found that Ubr1, similarly to Ufd4, contains a domain that specifically binds to N-terminal Ub but not to free Ub. *S. cerevisiae* lacking the Ufd4 component of the Ubr1-Ufd4 complex retained the Arg/N-end rule pathway but its proteolytic activity was lower than in wild-type cells. This could be seen not only with Arg/N-end rule substrates (i.e., substrates containing N-degrons) but also with Cup9, a transcriptional repressor of peptide import that is targeted by Ubr1 through an internal degron of Cup9. These and other results unified two proteolytic systems that have been studied separately over two decades (Hwang *et al*., 2010).

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147. Ubiquitin ligases of the N-end rule pathway: functional assessment of mutations in *UBR1* that cause the Johanson-Blizzard syndrome

Cheol-Sang Hwang[1], Maja Sukalo[2], Olga Batygin[1], Marie-Claude Addor[3], Han Brunner[4], Antonio Perez Aytes[5], Julia Mayerle[6], Alexander Varshavsky[1], Martin Zenker[2]

Johanson-Blizzard Syndrome (JBS) is an autosomal recessive disorder that includes congenital exocrine pancreatic insufficiency, multiple malformations such as nasal wing aplasia, and frequent mental retardation. Our previous work (Zenker *et al*. (2005) *Nat. Genet*. 37, 1345-1349) has shown that JBS is caused by mutations in human *UBR1*, which encodes one of E3 ubiquitin ligases of the Arg/N-end rule pathway (see Introduction). Most JBS-causing alterations of UBR1 result from nonsense, frameshift or splice-site mutations that are either certain or likely to completely abolish UBR1 activity. We report novel missense changes of *UBR1* in patients with milder variants of JBS. These changes and one previously reported missense mutation involve amino acid residues that are conserved between the 220-kDa human UBR1 and the 225-kDa Ubr1 of the yeast *Saccharomyces cerevisiae*. Taking advantage of this evolutionary conservation, we constructed alleles of yeast *Ubr1* that were counterparts of JBS-causing *UBR1* alleles, and found that the corresponding Ubr1 proteins were either functionally impaired or virtually inactive. These results,

inactive. These results, made possible by modeling mutational lesions of a human protein in its yeast counterpart, indicate that JBS is caused by either inactivation or a strong functional impairment of UBR1.

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Molecular, Cellular and Integrative Neuroscience

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Summary: Our laboratory investigates the psychological and neural bases of social cognition, using a number of different approaches. Some studies focus on the psychological level, using behavioral data from healthy people to make inferences about how emotion modulates memory, attention, or conscious awareness. A second approach uses neuroimaging and electrophysiology to investigate the neural mechanisms behind emotional and social processing. 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. Dr. Lynn Paul leads this work. In collaboration with Joe Piven at the University of North Carolina, we have also been studying people with autism. At the University of Iowa, we have ongoing collaborations that involve neurological populations with focal brain lesions, and, together with hospitals in the Los Angeles region, which involve neurosurgical patients in whom we can record intracranially.

A major focus in the past year has been on making comparisons across some of these populations and approaches. For instance, we are comparing people with autism and with amygdala lesions tested on the same tasks. Many of these comparative studies build on years of data accrual in our laboratory involving a significant amount of work by our staff, as well as the graduate students and post-docs. A second area where we are making comparisons is across methods. For instance, we are comparing responses measured in the amygdala to features of faces, and doing so using both the signal typically measured in fMRI studies (the BOLD response), as well as recording action potentials from single neurons in neurosurgical patients who have depth electrodes in the amygdala. Finally, we are continuing to collaborate with colleagues in the social sciences at Caltech who bring a model-based approach to understanding human behavior. Taken together, these studies of social cognition across a variety of populations, using multiple measures, and complemented with computational modeling, are giving us powerful insights not only into how specific structures might work (like the amygdala), but also how they might function in a network of multiple components. Extending our understanding of social cognition to the systems level, and examining the connections between different brain regions, constitutes a major thrust for future studies in our laboratory.

248. Lesion mapping of general intelligence

Jan Glaescher, Daniel Tranel, Hanna Damasio, Lynn Paul, David Rudrauf, Ralph Adolphs

General intelligence (*g*), a construct first proposed by Spearman in 1904, captures the performance variance shared across cognitive tasks and correlates with real-world success. Yet it remains debated whether *g* reflects the combined performance of brain systems involved in these tasks, or draws on specialized systems mediating their interactions. We investigated the neural substrates of *g* in 241 patients with focal brain damage using voxel-based lesion-symptom mapping, an approach that we already highlighted in a study last year. This mapping essentially contrasts the performance scores on some task between all those patients whose lesion includes a gives voxel, compared to all those patients whose lesion does not include that voxel. That voxel-wise statistic is then corrected across the entire brain using methods for multiple comparisons similar to what is used in fMRI studies (false-discovery rate correction). In this study, we used a hierarchical factor analysis across multiple cognitive tasks in order to derive a robust measure of *g*. Statistically significant associations were found between *g* and damage to a remarkably circumscribed albeit distributed network in frontal and parietal cortex, critically including white matter association tracts and frontopolar cortex (Figure 1). We suggest that general intelligence draws on connections between regions that integrate verbal, visuospatial, working memory, and executive processes.

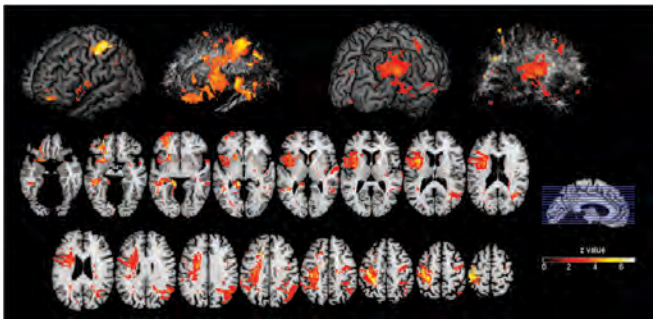

Figure 1. Lesion mapping of *g*. 3D renderings show cortical and subcortical regions with a statistically significant relationship between lesion location and *g* (corrected at 5% false discovery rate). Axial slices are shown for a more detailed inspection (Glaescher *et al.*, 2010).

249. Eye fixations following amygdala lesions

Daniel Kennedy, Ralph Adolphs

SM is a patient with complete bilateral amygdala lesions (cf. Figure 3) who fails to fixate the eyes in faces and is consequently impaired in recognizing fear. We reported several studies on her in the previous year and have continued our studies with this rare individual this year. In a series of studies that used an eyetracker to measure where people look when they see a face, we first replicated earlier findings in SM of reduced gaze to the eyes when seen in whole faces. Examination of the time course of fixations revealed that SM's reduced eye contact is particularly pronounced in the first fixation to the face, and less abnormal in subsequent fixations. In a second set of experiments, we used a gaze-contingent presentation of faces with real-time eye-tracking, wherein only a small region of the face is made visible at the center of gaze. In essence, viewers explore the face by moving a small searchlight over the face with their gaze. Under such viewing conditions, SM's fixations to the eye region of faces became entirely normalized (Figure 2). We suggest that this effect arises from the absence of bottom-up effects due to the facial features, allowing gaze location to be driven entirely by top-down control. Together with SM's failure to fixate the eyes in whole faces primarily at the very first saccade, the findings suggest that the saliency of the eyes normally attract our gaze in an amygdala-dependent manner. Impaired eye gaze is also a prominent feature of several psychiatric illnesses in which the amygdala has been hypothesized to be dysfunctional, and our findings and experimental manipulation may hold promise for interventions in such populations, including autism and fragile X syndrome.

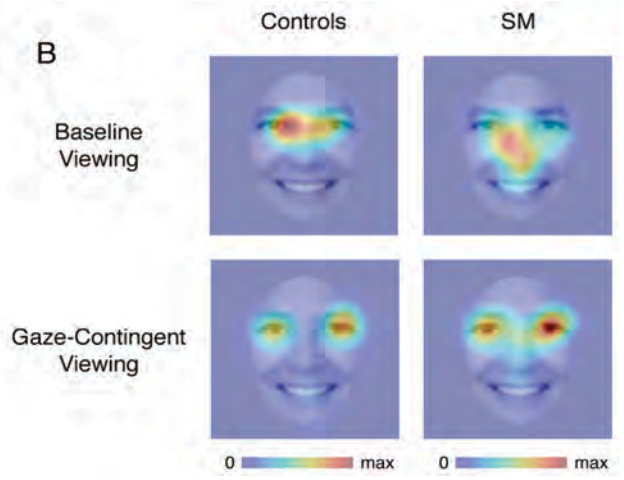

Figure 2. Density of fixations made to faces by a patient with bilateral amygdala lesions (SM) and matched controls. The top row shows results when viewers look at whole faces on a screen: whereas, controls look a lot at the eyes, SM does not. The bottom row shows the results from the gaze-contingent viewing, where people only see a small part of the face directly at their center of gaze. Under this condition, SM looked as much at the eyes as did the controls.

250. Comparing amygdala lesions and autism

Lynn K. Paul, Christina Corsello, Daniel Tranel, Ralph Adolphs

A leading neurological hypothesis for autism postulates amygdala dysfunction. This hypothesis has considerable support from anatomical and neuroimaging studies, as well as from studies in monkeys with amygdala lesions. People with bilateral amygdala lesions show impairments in some aspects of social cognition. These impairments bear intriguing similarity to those reported in people with autism, such as impaired recognition of emotion in faces, impaired theory of mind abilities, failure to fixate eyes in faces, and difficulties in regulating personal space distance to others (cf. Abstract 2). Yet such neurological cases have never before been assessed directly to see if they meet criteria for autism spectrum disorders (ASD). Here we undertook such an investigation in two rare participants with developmental-onset bilateral amygdala lesions.

We administered a comprehensive clinical examination, as well as the Autism Diagnostic Observation Schedule (ADOS), the Social Responsiveness Scale (SRS), together with several other standardized questionnaires. These are all well validated instruments regularly used to diagnose and assess autism. Results from the two individuals with amygdala lesions were compared with published norms from both healthy populations, as well as from people with ASD. Neither participant with amygdala lesions showed any evidence of autism across the array of different measures.

The findings demonstrate that amygdala lesions in isolation are not sufficient for producing autistic symptoms. We suggest instead that it may be abnormal

connectivity between the amygdala and other structures that contributes to autistic symptoms.

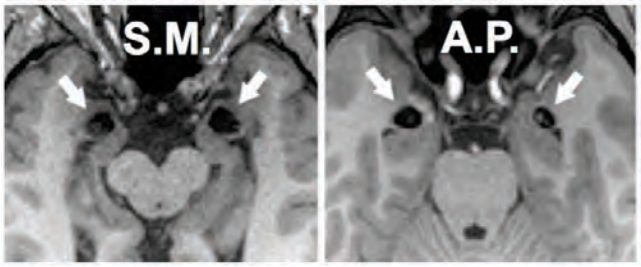

Figure 3. MRI scans of two patients with bilateral amygdala lesions. These individuals are also featured in abstracts 2 and 4. The arrows point to the two amygdalae, which are bilaterally lesioned. Both patients are women who have Urbach-Wiethe disease, an extremely rare genetic disorder resulting from a mutation in the gene that codes for extracellular matrix protein 1. In SM's case, the disease resulted in complete amygdala lesions, in AP's the lesions encompass approximately half the amygdala on both sides of the brain.

251. No fear of losing money in patients with bilateral amygdala lesions

Benedetto deMartino, Colin Camerer, Ralph Adolphs

Losses are an inevitable possibility of many risky decisions, and organisms have evolved mechanisms to minimize their likelihood. People avoid the risk of losses even when faced with the chance of acquiring a substantially larger gain, a behavioral preference termed "loss aversion." The cautionary brake on behavior known to rely on the amygdala is a plausible candidate mechanism for loss aversion, yet evidence for this idea has so far not been found. We studied two rare individuals with focal bilateral amygdala lesions (SM and AP shown in Figure 3) using a detailed series of experimental economics tasks. In order to test individual sensitivity to financial losses we asked participants to play a comprehensive series of mixed (gains and losses) monetary gambles of variable magnitude. Participants saw a coin-flip gamble that offered equal (50%) chances of gaining or losing different amounts of money (corresponding to the squares in the figures below). While both participants retained a normal ability to respond to changes in the gambles' expected value and risk (that is, they did prefer winning over losing, and they preferred a sure amount rather than a risky amount), they showed a dramatic reduction in loss aversion compared to controls. Both of them accepted gambles that no control subject took. The findings suggest that the amygdala plays a key role in generating loss aversion by inhibiting actions with potentially deleterious outcomes.

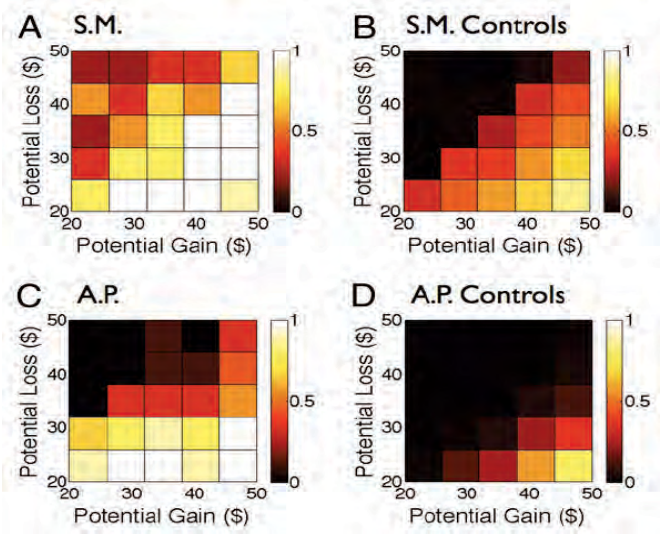

Figure 4. Amygdala Lesions Block Loss Aversion. Color-coded heatmaps depict the probability of gamble acceptance at each level of gain/loss (white indicates high willingness to accept the gamble, and black indicates low willingness to accept the gamble) within that cell. S.M. and A.P. (A and C), two patients with bilateral amygdala lesions (cf. Figure 3) were noticeably less loss averse than their respective control groups (panels B and D, respectively).

252. Violations of social distance norms in autism

Daniel Kennedy, Ralph Adolphs

Autism spectrum disorders (ASD) feature a profound impairment in social functioning that has been the topic of intense research efforts. Yet one component that contributes importantly to real-life functioning has been relatively neglected in research to date: the ability to appropriately regulate one's distance from other people during social interactions. The purpose of the present investigation was to test the hypothesis that people with ASD have impaired social distance regulation.

Parent-report data on the Social Responsiveness Scale (SRS) were obtained from two large extant datasets (the Autism Genetic Research Exchange and the Simons Simplex Collection), in order to compare ASD probands and their unaffected siblings on parent-reported ratings of social distance competency. The SRS is a lengthy questionnaire that asks the parent (often the mother) questions about the early childhood history of the autistic child. After applying exclusionary criteria, our final sample consisted of 766 ASD probands and 766 of their unaffected siblings. All participants were between the ages of 4-18. ASD probands were rated as being less aware of social distance than their unaffected siblings. This difference arose from several specific items on the SRS, such as item #55, which asks, "Knows when he or she is too close to someone, or is invading someone's space," as well as several other items that were highly correlated with item #55 and that also ask questions related to social distance. Moreover, abnormality of social

distancing showed a specific correlation with scores on the social and communication subscales of the Autism Diagnostic Interview - Revised (ADI-R), a gold-standard diagnostic instrument. Abnormal social distance regulation thus, appears to be a pervasive feature of ASD. It is likely that this abnormality contributes significantly to the social impairments experienced in real life by people with ASD. Of interest, our Biology Annual Report from last year noted a study in the amygdala-lesioned patient SM (Kennedy *et al*., 2009) that found highly abnormal social distance regulation in this patient. It is thus, possible that abnormal social distancing in autism is related to amygdala dysfunction.

253. Autobiographical memories of emotional moral actions

Jessica Escobedo, Ralph Adolphs

Our autobiographical self depends on the differential recollection of our personal past, notably including memories of morally laden events. While both emotion and temporal recency are well known to influence memory, very little is known about how we remember moral events, and in particular about the distribution in time of memories for events that were blameworthy or praiseworthy. To investigate this issue in detail, we collected a novel database of 758 confidential, autobiographical narratives for personal moral events from 100 well-characterized healthy adults. Each participant came into the lab for several hours and first narrated autobiographical memories in response to specific cue words (the words listed on the x-axis of Figure 5). These were then transcribed and later rated on a number of measures, including how recent the memory was, and how negative or positive it was. The ratings of pleasantness were corroborated in an independent group of 50 raters. We found that negatively valenced moral memories (morally reprehensible acts that one had done) were significantly more remote than positively valenced memories (morally praiseworthy acts), both as measured by the valence of the cue word that evoked the memory, as well as by the content of the memory itself (Figure 5). The effect was independent of chronological age, ethnicity, gender, or personality, arguing for a general emotional bias in how we construct our moral autobiography. The results argue for a strong effect of temporal remoteness on the valence of recollected moral memories, independently of any other factors: we remember our best deeds as the most recent ones, and our worst deeds as the most remote ones. The findings are consistent with several psychological theories of how we construct our autobiography.

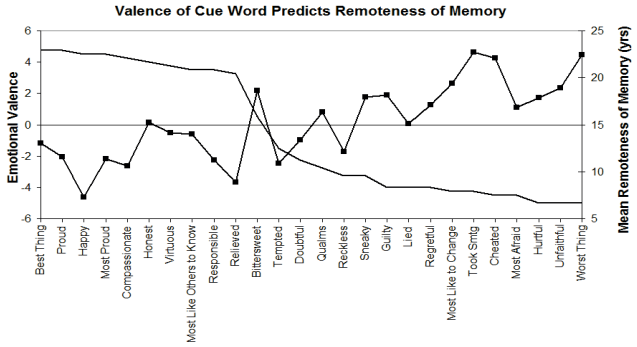

Figure 5. Negative moral memories are farther in the past than positive moral memories. From over 700 memories collected in 100 participants, we found that moral memories elicited by positively valenced cues are more recent, whereas, those elicited by negative cues are more remote. The cue words used are shown on the x-axis. Emotional valence of the cue words is associated with the age of the memories they evoke ($R=0.74$; $p=0.004$). Remoteness of memory is shown with solid square symbols; valence is plotted without symbols.

254. Feeling and recognizing emotions are correlated

Tony Buchanan, David Bibas, Ralph Adolphs

How do we recognize emotions from other people? One possibility is that our own emotional experiences guide us in the online recognition of emotion in others. This idea, in line with modern "simulation theory," essentially proposes that we put ourselves in the shoes of other people in order to figure out how they feel. A distinct but related possibility is that emotion experience helps us to learn how to recognize emotions in childhood, independently of any simulation mechanism. We explored these ideas in a large sample of people ($N = 4{,}608$) ranging from 5 to over 50 years old who visited a traveling exhibit of the California Science Center in Los Angeles. Participants were asked to rate the intensity of emotional experience in their own lives, as well as to perform a task of facial emotion recognition. Those who reported more intense experience of fear and happiness in their lives were also significantly more accurate in recognizing facial expressions of fear and happiness, respectively, and intense experience of fear was associated also with more accurate recognition of surprised and happy facial expressions (Figure 6). The associations held across all age groups. These results suggest that the intensity of one's own emotional experience correlates with the ability to recognize emotions in others, and demonstrate such an association already by age 5. This is the first report of such a correlation, and it is consistent with simulation theory accounts of how we recognize other people's emotions.

Figure 6: Feeling emotions and recognizing them from faces are correlated. Data from 4,608 participants (means and SD) is shown in the case of fear. On the y-axis is a measure of the accuracy in recognizing fear in faces (participants had to move a slider around to slowly morph a face into what they thought was the prototype for fear, so lower values indicate a more accurate representation of fear expressions). On the x-axis are the self-ratings that participants endorsed in terms of the average intensity of fear that they personally have experienced in their own lives, Buchanan *et al.*, 2010.

255. Use of information from the eyes in faces following unilateral amygdala lesions

Frederic Gosselin, Michael Spezio, Daniel Tranel, Ralph Adolphs

The human amygdalae are involved in processing visual information about the eyes within faces (cf. Abstract 2), and play an essential role in the use of information from the eye region of the face in order to judge emotional expressions, as well as in directing gaze to the eyes in conversations with real people. However, the roles played here by the left and right amygdala individually remain unknown. Here we investigated this question by applying the "*Bubbles*" method, which asks viewers to discriminate facial emotions from randomly sampled small regions of a face. On each trial, participants see only small pieces of a face, and are asked to discriminate fear from happiness. By regressing the accuracy of the responses on the locations of the face that was revealed in each trial, we were able to extract the "effective information" that viewers use from faces in order to discriminate emotions. We administered this task to 23 neurological participants with focal, unilateral amygdala damage (10 to the right amygdala). We found a statistically significant asymmetry in the use of eye information when comparing those with unilateral left lesions to those with unilateral right lesions. Specifically, there was a statistically significant difference in use of the left over the right eye between the group with left amygdala lesions compared to the group with right amygdala lesions (Figure 7). This difference was due to a preferential shift towards processing information from the eye region on the side of the image ipsilateral to the intact amygdala (contralateral to the lesion). These effects were found only when participants made fearful versus happy emotion discriminations, and not when they were asked to discriminate gender. The findings have implications for the amygdala's role in emotion recognition and gaze direction during face processing.

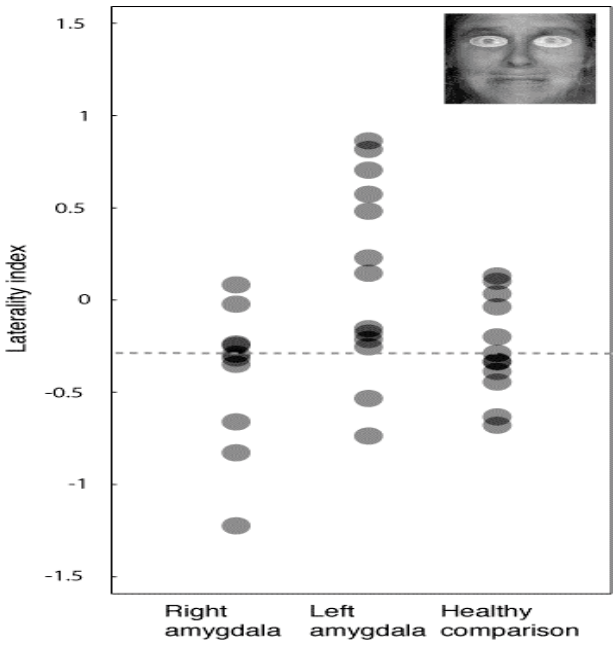

Figure 7. Difference in use of information from the right eye and the left eye. We plot a laterality index as the peak classification image value in the eye region on the left side of the image (from the viewer's perspective) minus the region on the right side of the image in individual participants. Each amygdala lesion group showed a bias in favor of using information from that side of the face ipsilateral to their intact amygdala, resulting in a statistically significant difference in the laterality index between the two groups ($p<0.01$, two-tailed). The inset shows the left and right eye regions as bright blobs superimposed on the average of the four facial expression photos used in the experiment. These were used as regions of interest to derive the difference in use of information from the eye displayed on the right and left side of the face.

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Research and Laboratory Staff: Atiya Hakeem

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Summary: We are continuing to explore the structure and function of the von Economo neurons in health and disease and have found evidence that they express many cytokine receptors, which is consistent with a possible role for the anterior cingulate and fronto-insular cortices, which contain these specialized neurons, in neuro-immune interactions (**Figure 1**). We are analyzing the comparative structure of the frontal lobe in primates using connectivity based on high resolution diffusion tensor magnetic resonance imaging, and cellular and fiber architecture (**Figure 2**). Finally we have initiated a study of the brains of non-demented centenarians using these same methods as part of an endeavor to understand brain aging in extremely old individuals who had preserved functioning (**Figure 3)**.

256. A potential tole of the VENS and fork cells in the regulation of immune functions

Tetreault, N.A., Hakeem, A.Y., Stimpson, C.D[1]., Jacobs, B.[2], Sherwood, C.C.[1], Wold, B.J., Allman, J.M.

Von Economo neurons (VENs) are large bipolar cells that are a specialized feature of the brains of apes and humans. The VENs are primarily located in anterior cingulate cortex (ACC) and frontoinsular cortex (FI), regions of the brain associated with regulation of physiological states in the body, error recognition, and decision-making. There are several lines of evidence that the VENs and other related layer 5 neurons are involved in immune function and regulation. Our previous study of gene expression using RNA-Seq in FI autistic and control subjects indicated the likely involvement of this area in immune functioning (Tetreault *et al.*, SFN, 2009). In the present study, we immunocytochemically stained ACC and FI in normal adult brains and found VENs and related layer 5 neurons (fork cells) were preferentially labeled by antibodies to activating transcription factor 3 (ATF3), the interleukin receptors IL1R1, IL1-beta, IL4R1-alpha, IL6R, IL8R-beta, IL10R-beta, oxidized lipoprotein receptor (OLR1) and suppressor of cytokine signaling (SOCS3). Using Ingenuity Pathway Analysis (IPA) we have found a potential protein interaction network for the VENs suggesting a possible role in immune regulation in the brain. Additionally, we found that VENs are preferentially labeled by ATF3 and IL4R antibodies in humans and apes. ATF3 is a transcription factor implicated in nerve regeneration after injury. I L1B is a cytokine that is activated early in inflammation. IL4R-alpha, IL6R, and IL10R-beta and SOCS3 are involved in various aspects of immune regulation, suggesting that the VENs participate in these functions. Increasing serum concentrations of IL6 are linearly related to slower reaction times in the Stroop task, which measures the subject's capacity to discount distracting stimuli and is strongly linked to the activity of dorsal ACC (Brydon *et al.*, 2008). The induction of negative mood by immune activation is related to increased activity in subgenual ACC (Harrison *et al.*, 2009). Negative mood also directly activates subgenual ACC and leads to the increased production of inflammatory cytokines measured peripherally in the saliva (O'Connor *et al.*, 2009). The activity of subgenual ACC is also linked to two measures of the severity of asthmatic response when provoked by allergens under carefully controlled conditions (Rosenkrans and Davidson, 2009). Thus, there are potential linkages between the activity of ACC, the inflammatory and anti-inflammatory cytokines whose receptors are strongly expressed in the VENs and related neurons, slowed cognitive functioning, the induction of negative mood, and immune system function and dysfunction.

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257. Histology and diffusion-tensor imaging study of the brain of a 104-year-old human

Soyoung Park, J. Michael Tyszka, Anastasia Markovtsova, Ralph Adolphs, John M. Allman

We have acquired the right hemisphere of a 104-year-old woman's brain from the NICHD Brain and Tissue Bank for Developmental Disorders. The subject had been in good health and showed only mild cognitive slowing until her death. The hemisphere was scanned in the Bruker 7-Tesla magnet at the Caltech Brian Imaging Center. This yielded a high-resolution structural magnetic resonance image with a voxel size of 0.275 mm and a diffusion-tensor image (DTI) with a voxel size of 0.8 mm. The hemisphere was then cut coronally into five blocks, and so far the two most anterior blocks have been sectioned coronally at the approximate thickness of 140 microns for histological staining.

The structural image of the hemisphere showed a large number of small dark patches (FIGURE 1)

distributed throughout the substantia nigra, caudate nucleus and the putamen. These dark patches are probably the result of signal capture by iron deposits. Many of the neurons in the substantia nigra are dopaminergic and send their projections to the caudate and putamen. Iron is co-localized with dopamine in neurovesicles in the soma and axons of these cells (Ortega *et al*, 2007); however these dark patches are not typically seen in these structures in the MRIs of younger subjects. Since elevation in the iron level in the brain is implicated in normal aging, as well as pathologies such as Parkinson's disease and Alzheimer's disease (Hallgren and Sourander, 1958; Dexter *et al*., 1989; Bartzokis *et al*., 2000), we plan to investigate this matter further, in particular using the Perls' Prussian blue stain for ferric iron, Iba-1 immunostain for microglia – which are known to sequester iron – and the Bielschowsky stain for senile plaques and tangles.

The DTI data and the Gallyas staining of myelin suggested selective degeneration of myelin sheaths in the tissue. From the fractional anisotropy (FA) map of the hemisphere, we observed signs of disorganization and atrophy in the corpus callosum. This led us to suspect that there might be abnormalities in the axons. In low magnifications in sections stained with the Gallyas method, the axon bundles look normal. However, higher-magnification views showed fragmentation of myelin sheaths of the fibers. This is clearly not due to postmortem changes, since the post-mortem interval to fixation was only 4 hours and we have done Gallays staining in many brains without seeing myelin fragmentation. Instead, we attribute this to the subject's extreme old age, since myelin fragmentation has been observed in the brains of very old macaque monkeys (Peters, 2002).

On the other hand, the FA map displayed the cingulum bundle as robust and highly organized, hence suggesting that the myelin degeneration may be regionally selective. To further investigate the relationship between myelin sheath fragmentation and aging, we plan to measure the diffusivity of various regions of the hemisphere using the DTI data, in addition to performing immunohistochemistry for proteins associated with myelin breakdown.

We have recently received another centenarian brain hemisphere: a 101-year-old male who neurologically normal and described as having an excellent memory. We will process the tissue the same way we prepared the 104-year-old hemisphere (imaging in the 7-T magnet, sectioning, and histochemistry) and explore the iron deposit and fiber integrity. Our plan is to continually expand our number of elderly brain samples and hopefully gain an insight into the process of neurological aging in healthy, non-demented, extremely elderly subjects.

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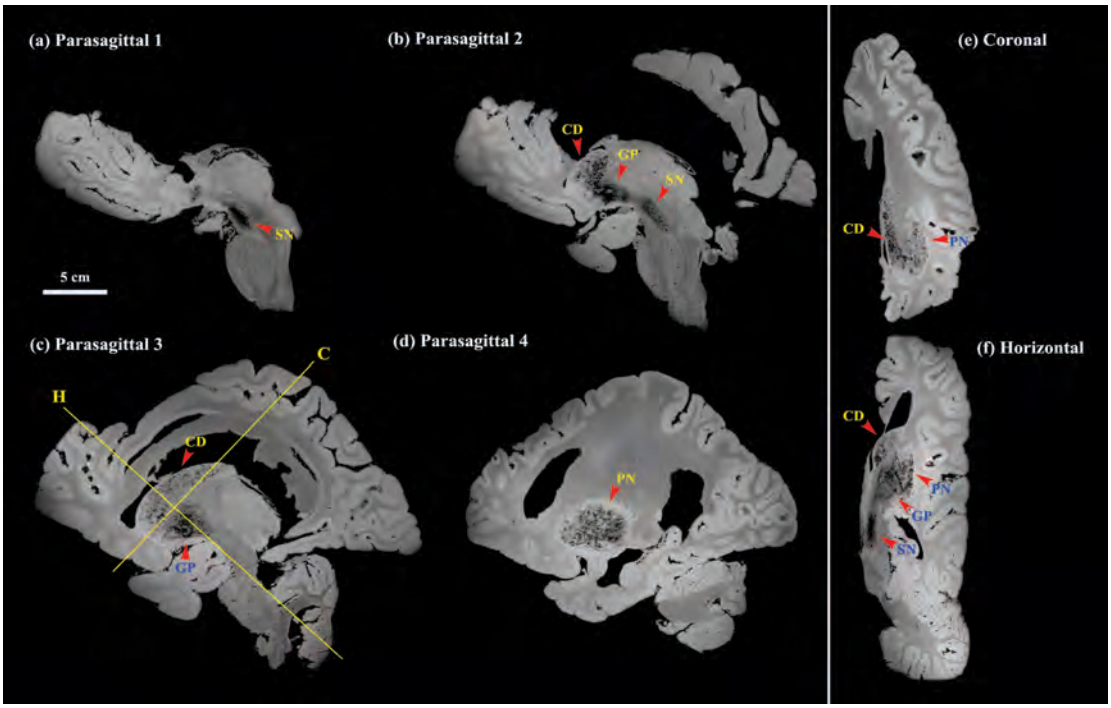
Figure 1



258. Histology and diffusion-tensor imaging study of the *Microcebus murinus* claustrum and insula

Soyoung Park, J. Michael Tyszka, John M. Allman

We have examined the structures and connectivity patterns of the claustrum and the insula in the brain of *Microcebus murinus*, the gray mouse lemur, using diffusion-tensor imaging (DTI) and histological methods for staining cell bodies and myelin. The right hemisphere of the brain was scanned with the Bruker 9.4-Tesla magnet at the Caltech Brain Imaging Center. The scan yielded a very high-quality diffusion-tensor image with a voxel size of 0.09 mm. This particular hemisphere was then used for a separate project, but the whole brain of another *Microcebus murinus*, which was sectioned coronally at the approximate thickness of 0.09 mm for histological staining. Every odd-numbered section was stained using the Nissl method for visualizing neuronal cell bodies and glia, and every even-numbered section underwent the Gallyas method for staining axon fibers.

Upon inspecting the sections stained with the Nissl and Gallyas methods, we found that the claustrum and the insula are extremely close together in the *Microcebus* brain (Figure 1). This is not the case in the brains of other primates, in whose brains these two structures are well separated by the extreme capsule. *Microcebus* is thought to have retained primitive features present to the common ancestor of all primates. Hence we hypothesize that, in the common ancestor of primates, the claustrum and the insula started out as close and highly connected structures, but the claustrum gradually drifted apart and formed an island during evolution. A recent immunohistochemical study on the rat and monkey showed that the claustrum is fully surrounded by neurons that are found in a deep layer of the insula, supporting this hypothesis (Mathur *et al*., 2009). In addition, according to Rose-Wilno's study on the ontogeny of the human brain, claustrum and insula are not fully separate at the age of 2-2.5 months (Edelstein and Denaro, 2003).

The fiber tractography in the insula yielded results that are distinct from the connectivity pattern of the claustrum (Figure 2). In fact, considering the proximity of the two structures, it is remarkable how different their connectivity patterns are. The most prominent connections for the insula are with the cingulate and dorsal prefrontal cortex with additional connections with the thalamus and brain stem. The most prominent connections of the claustrum are with the ventral prefrontal cortex, the olfactory cortex and bulb, and the entorhinal cortex.

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Figure texts:

Figure 1: Visualizations of the *Microcebus murinus* brain using histology and magnetic resonance imaging. (A) is the photomicrograph of a coronal section of the brain, stained for cell bodies using the Nissl method; (B) is the photomicrograph of a section adjacent to the section shown in (A), stained for myelin using the Gallyas method; (C) is a coronal view of the DTI data of the brain, with probabilistic fiber tracking results superimposed upon the image: The red-yellow signal indicates the results from the claustrum, and the blue-light blue signal is from the insula. The more probable signals are shown in yellow or light blue, whereas less probable connections are displayed in red or darker blue. The regions in which the signals from the claustrum and the insula overlap are shown in purple; the seeds for the claustrum (CL) and insular (IN) tractographies are indicated by the small black squares; (D) is a coronal view of the structural magnetic resonance image of the brain.

Figure 2: Probabilistic fiber tractography results of the claustrum and the insula in the *Microcebus murinus* brain, shown in four parasagittal planes (A, B, C and D plotted against the same coronal sections illustrated in Figure 1). Tracts arising from the seed placed in the claustrum are plotted in red; tracts from the insula seed are in blue.

Figure 2

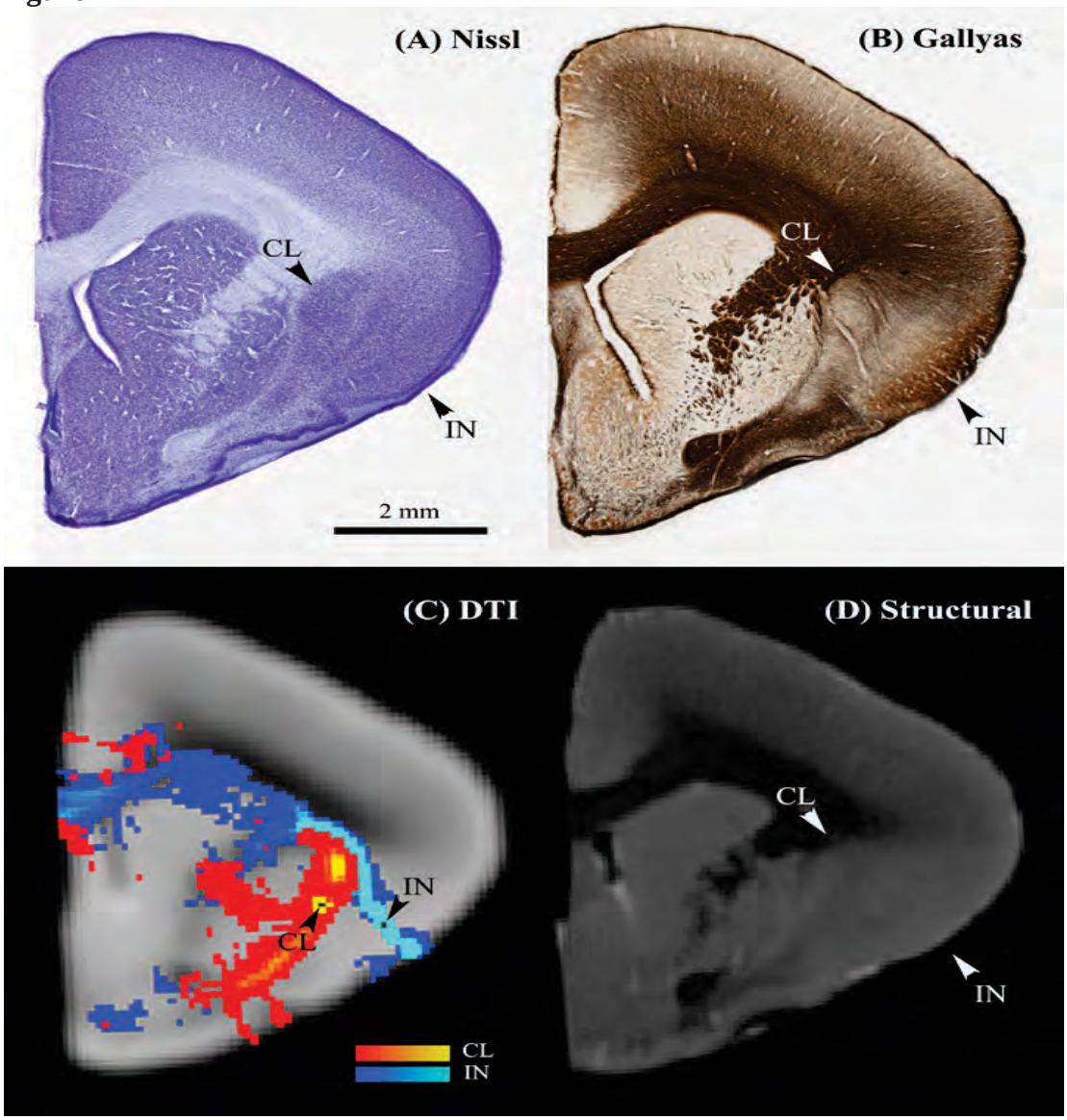
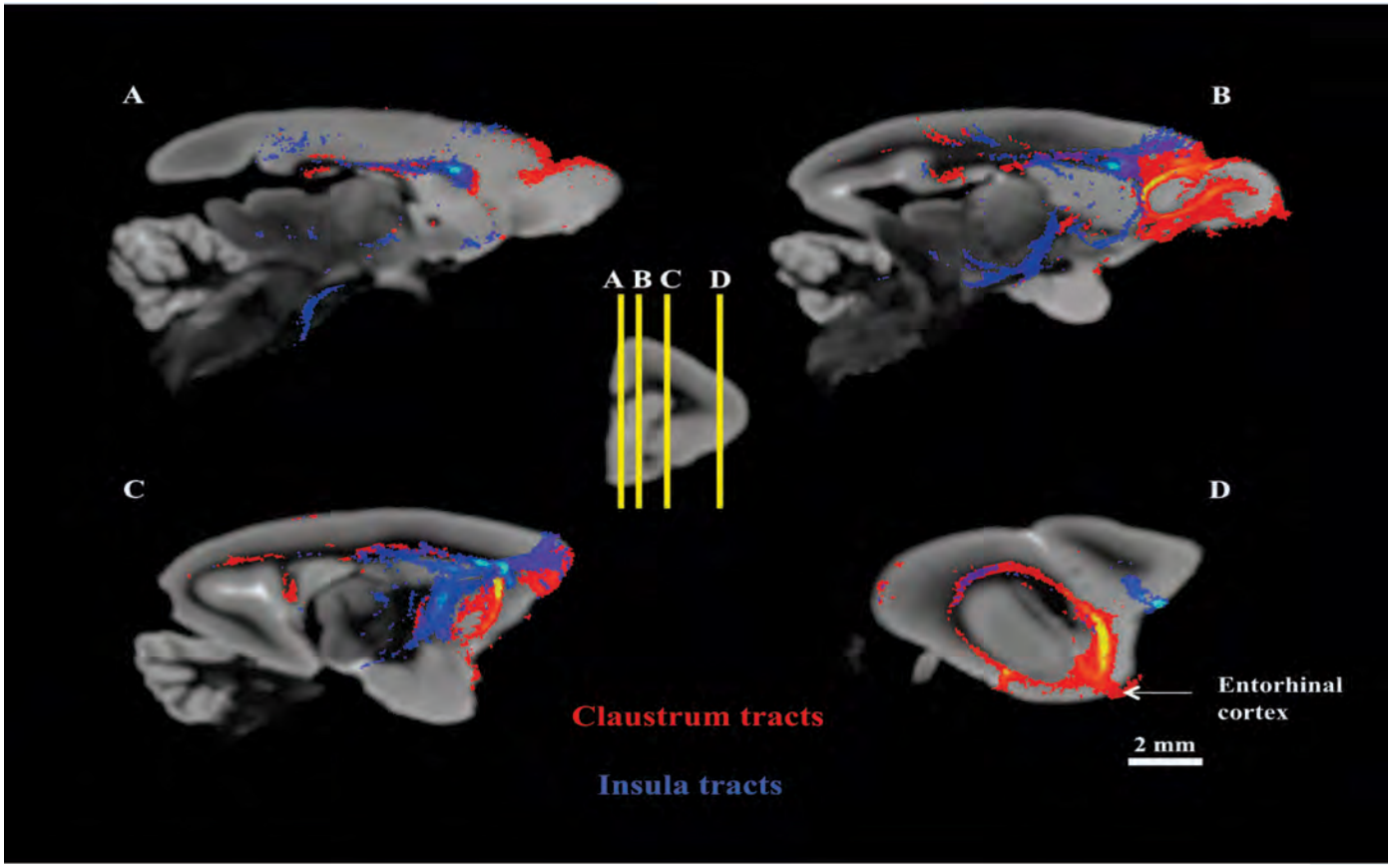

(A) Nissl
(B) Gallyas
CL
IN
2 mm
CL
IN
(C) DTI
(D) Structural
IN
CL
CL
IN
CL
IN

Figure 3



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Allman, J.M., Tetreault, N.A., Hakeem, A.Y., Manaye, K.F., Semendeferi, K., Erwin, J.M., Park, S., Goubert, V. and Hof, P.R. (2010) The von Economo neurons in frontoinsular and anterior cingulate cortex in great apes and humans. *Brain Struct. Funct.* **214**:495-517.

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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 reward 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.

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 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 torques and 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 that involve the movements of multiple body parts such as bimanual movements.

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.

259. Multiple reference frames for reaching in parietal area 5d

L.R. Bremner, R.A. Andersen

The execution of reaches to visual targets requires coordinate transformations between the neural representations of the sensory signal (in which targets are initially coded retinotopically) and the eventual motor command (in which targets are coded with respect to the effector limb). In macaques, anatomically connected circuits between the posterior parietal cortex and frontal cortex have been shown to play a role in this aspect of reach planning. Neurons in the parietal reach region (PRR) represent the location of planned reach targets (T) in a gaze-centered reference frame (T-G; ref. 1), neurons in dorsal area 5 (area 5d) of parietal cortex represent targets with respect to both the gaze location and the initial hand position (T-G, T-H; ref. 2), and dorsal premotor cortex (PMd) is known to contain a complete set of vectors for relative coding of the hand, gaze and target positions (T-G, T-H, H-G; ref. 3).

We sought to extend these findings by examining whether area 5d also shows complete relative coding. We present spike data from one rhesus macaque during a delayed reaching task in which the positions of the hand, gaze and target were systematically varied from -20 deg of visual angle to +10 deg. Preliminary data confirm the previous finding (ref. 2) that area 5d represents the reach targets with respect to both gaze location and initial hand location, but indicate the absence of a full relative code at the population level. At the single cell level, a large degree of heterogeneity was observed: a small group of cells (~8%) did encode all three vectors but almost a third of the recorded cells showed purely hand-centered or purely gaze-centered coding of target positions. A further 10% showed firing patterns consistent with a representation of the target in a head or body-centered reference frame (although this was not explicitly tested in this experiment). This heterogeneity is consistent with the range of visual, proprioceptive, and efference copy signals which area 5 receives. Ongoing experiments are being performed to address hand and gaze position coding in this area across a wider range of locations in space.

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260. Effects of visual stimulation on LFPs and spikes in PRR

E. Hwang, R.A Andersen

A growing body of evidence suggests that local field potentials (LFPs) follow the average membrane potentials, largely reflecting synaptic inputs of nearby neurons, while spikes are the output of nonlinear operations (e.g., threshold and saturation) on the membrane potential of a single neuron. Thus, LFPs are likely to encode, in part, common input signals that a number of neighboring neurons share while losing input (membrane potential) specificity for individual neurons from averaging across many neurons.

A previous study from our lab found that two subpopulations of neurons, class V and M, are intermingled with similar proportions in the parietal reach region (PRR). Only class V showed spiking activity for visual stimuli and thus, may receive strong input from extrastriate cortex, while both classes showed spiking activity tuned to the direction of planned movements. Since a significant proportion (46%) is class V, visual stimulation altering the membrane potentials of class V neurons could affect not only their spiking activity but also LFPs in PRR.

Here we tested these predictions by examining the changes of the LFP and spiking activity in PRR induced by visual stimulation during the reach planning period. A visual stimulus at the reach goal disappeared during the planning period in the memory-guided reach task, whereas it remained illuminated in the vision-guided reach task. Thus, visual stimulation effects on LFPs and spikes could be inferred by comparing their planning period activity between the two conditions.

We found that: 1) the LFP power in the gamma bands (above 40 Hz) was larger and more strongly modulated by the impending reach goal (or visual stimulus location) during the planning period in the vision-guided reach than the memory-guided reach, regardless of the class of the simultaneously recorded neurons on the same electrode; 2) the firing rate was higher in the vision-guided reach than the memory-guided reach only for class V neurons; and 3) the LFP gamma band power was equally correlated with the firing rate of the two classes in the memory-guided reach but it was better correlated with class V than M in the vision guided reach. Therefore, the visual stimulation affected the gamma band LFPs and the firing rate of class V in a correlated manner, suggesting that the synaptic inputs from the visual cortex induced a significant synchronization of the membrane potentials of class V neurons in the gamma bands.

261. Interhemispheric interactions in parietal cortex during spatial decision making

I. Kagan, M. Wilke, R.A. Andersen

The circuitry underlying visuomotor decisions encompasses a bihemispheric network, in which each hemisphere is thought to represent mainly contralateral targets and response options. Several models and empirical data postulate inter-hemispheric competition and/or cooperation, but how the signals from the two hemispheres interact and how these signals are integrated to elicit an action is not known. We investigated intra- and interhemispheric processing during spatial decisions between alternative response options in opposite visual hemifields. Using a delayed oculomotor decision task with event-related fMRI in monkeys we found that several frontoparietal areas, including LIP, exhibited activations consistent with interhemispheric competition. In particular, BOLD activity for the two-target choice trials was on average lower than in the single-target instructed trials. To study the interhemispheric interactions on the neuronal level we made bihemispheric multielectrode recordings of spiking activity and local field potential (LFP) in LIP, using the same task. Spiking and LFP exhibited contralateral tuning in the cue and delay intervals. In the LFP the tuning was present in the low (d-q-a-b) and in g frequencies of band-limited power (BLP). As in the BOLD signal, the spiking and LFP cue responses were ~15-25% less in two-target choice trials as compared to instructed trials, even when the selected target was in the response field. In addition, the BLP amplitude reflected the upcoming spatial choice.

The competition was also manifested on a trial-by-trial basis: spike rates co-varied in similarly tuned units in the same hemisphere but were mostly anti-correlated in interhemispheric pairs with non-overlapping response fields. Likewise, interhemispheric BLP correlations in oppositely tuned sites were near zero or negative. The LFP/BLP correlations and LFP-LFP coherence were significantly stronger within each hemisphere than between hemispheres. There were task-related differences between intra- and interhemispheric coherence. The intrahemispheric coherence was dominated by the 10-40 Hz range throughout the task, as well as in the inter-trial interval. In contrast, interhemispheric coherence was strong in 10-30 Hz range during the task, while the inter-trial coherence shifted to <10 Hz range. The enhancement in decision trials relative to instructed trials was present in <10 Hz range, in the interhemispheric but not in the intrahemispheric coherence. Taken together, BOLD, spiking and LFP activity patterns support the notion of competing push-pull interactions between response options, and dynamic coupling between the two hemispheres.

262. Cross-cortical communication via coherence

C.A. Stetson, R.A. Andersen

How do different brain areas communicate? From the earliest electrical recordings of the brain, it has been clear that brain areas synchronize differently depending on the brain's state. Recent evidence has shown individual action potentials (spikes) synchronizing with fine-scale rhythmic oscillations (local field potentials, or LFPs) within and across brain areas. But is this synchronization, or coherence, responsible for communication? A classic measure of communication is the mutual information. We here show that individual spikes in one cortical region tend to share more information with spikes in another region when they also cohere with LFPs recorded there. This relationship holds true over several linear, time-independent brain states, including sleep, idleness, and working memory, though these states have different characteristic frequencies. We recorded from brain regions known to be involved in action planning and decision-making -- the dorsal premotor region (PMd) and the medial bank of the intraparietal sulcus (MIP, part of the Parietal Reach Region). We show that when deciding whether to move with the hand or eye, cells that report the monkey's decision before his movement tend to cohere more cross-cortically than cells that make a later report. This evidence may suggest that during an internally generated decision, cells with something to say are the ones who do the talking.

263. An ultra-low-power 32-channel wireless neural recording interface

W. Wattanapantch[1], D. Kumar[1], B. DoValle[1], L. Turicchia[1], B.I. Rapoport[1,2], S.K. Arfin[1], E. Hwang, R.A. Andersen, R. Sarpeshkar[1]

Wireless, micropower, fully implantable microelectronic systems are essential for clinically viable neural prosthetic devices, and will enable investigators to probe the neural correlates of behavior in real time. Here we describe such a system and its performance in acute and chronic tests in awake, behaving rats and rhesus macaques. Our wireless neural recording system consists of three custom integrated circuits (ICs), a low-power field-programmable gate array (FPGA), surface-mounted components, and two concentric coils for separate data and power transfer, integrated onto a flexible printed circuit board (PCB). The first IC consists of 32 neural recording amplifiers, multiplexed onto eight analog-to-digital converters (ADCs). Each amplifier channel can be configured to record action potentials with a passband of 300-9100 Hz, or local field potentials (LFPs) with a passband of <1-300 Hz. The gain of each amplifier can be programmed in eight steps from 43 dB to 63 dB, and the input-referred noise in spike-recording mode is 5 V (RMS) while consuming 8 W of power. Each ADC, while consuming effectively 680 nW per channel, digitizes signals recorded from 4 amplifiers to 8-bit precision with an effective sampling rate of 31.25 kS/s per channel.

The second IC is a bidirectional impedance-modulation wireless data link that uses near-field inductive coupling at 25MHz to transmit digitized data wirelessly to an external receiving unit at up to 5.8 Mbps (uplink); it can also receive information, used to program the implanted unit, at up to 300 kbps (downlink). This IC is designed to minimize power dissipation by the implanted unit, which dissipates only 100 W. The third IC is an ultra-compact analog lithium-ion (Li-ion) wireless battery charger that achieves 75% power efficiency and charging voltage accuracy of 99.8% relative to the target 4.2 V. The FPGA processes data from the recording IC before sending it to the wireless telemetry IC. Downlink wireless programming enables selection of one of three recording modes: calibration, full-bandwidth data from 8 of the 32 channels, or full-bandwidth data from four channels and spike event timing from all channels. The FPGA also implements a finite-state machine that enables the system to operate either in one of the recording modes, a power-conserving mode, or a battery-recharging mode. The system is powered by a 3.7-V, 25-25 mAh Li-ion polymer battery. Clock signals are derived from surface-mounted crystal oscillators. The system has a footprint of 186 mm2 and a thickness of <4 mm. The coils used for wireless data and power telemetry added a 17-mm-diameter region to the total device area. The entire system consumes less than 6 mW.

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264. Pulvinar inactivation alters cortical responses during spatial decision making

M. Wilke, I. Kagan, R.A. Andersen

The neural mechanisms of sensorimotor transformations and spatial decision making have been extensively studied in cortical areas, but little is known about the role of thalamic structures such as the pulvinar in these processes. Several cortical areas known to be involved in these functions are reciprocally connected to the pulvinar, suggesting that it may play an important role in the coordination of visuomotor behavior (Asanuma *et al*., 1985; Gutierrez *et al*., 2000). Consistent with this hypothesis, a recent study has shown that dorsal pulvinar inactivation in rhesus macaques disrupts several aspects of visuomotor behavior, including spatial decision making (Wilke *et al*., 2010). The goal of the present study is to investigate the neural network changes that underlie the observed visuomotor and decision deficits and more generally, to understand the contribution of the pulvinar to cortical processing. To this end, we measured whole-brain BOLD activity in a 4.7T MRI scanner while monkeys performed memory saccades to either instructed (only one target) or autonomously chosen locations (choice between two targets). We compared event-related BOLD activity between control- and inactivation sessions in which we injected the GABA-A agonist THIP into the lateral portion of the pulvinar. After inactivation, memory saccade performance to single targets remained intact, but monkeys exhibited a significant choice bias towards targets in the ipsilesional hemifield. On the neuronal level, we observed three main effects of unilateral pulvinar inactivation that were both anatomically and functionally specific. (1) Activity was reduced in distant but anatomically connected cortical areas within the injected hemisphere. Specifically, in the instructed (one target) condition, pulvinar inactivation reduced cue and delay period BOLD activity in several areas in the posterior parietal cortex (e.g., LIP) and the superior temporal sulcus (e.g., TPO); (2) The same temporo-parietal areas in the intact hemisphere exhibited 'hyperactivity' when an ipsilesional cue was presented; and (3) In the choice (two targets) condition, we observed a compensatory increase of activity in both hemispheres when monkeys chose targets in the contralesional ('neglected') hemifield. Taken together, these findings highlight the contribution of the lateral pulvinar to cortical activity underlying visuomotor transformations and spatial decision making. Observed fast compensatory changes in both injected and healthy hemispheres also suggest the dynamic recruitment of bihemispheric networks in overcoming unilateral thalamic dysfunction.

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Summary: Research in this laboratory is aimed at understanding the neurobiology of emotion. We seek to elucidate how fundamental properties common to emotional states or responses, such as arousal, are encoded in the circuitry and chemistry of the brain, and how sensory stimuli eliciting specific emotional responses, such as fear or aggression, are transformed and organized into behavior by the nervous system. Our work employs molecular genetic tools to mark, map and functionally manipulate specific circuits, to determine how identifiable populations of neurons contribute in a causal manner to behavior. These interventional studies are complemented by electrophysiology and functional imaging to measure activity in neural circuits. We use two model organisms in the laboratory: mice and the vinegar fly *Drosophila melanogaster*, with roughly equal emphasis on both.

Emotion circuits in the mouse brain

Research using the laboratory mouse *Mus musculus* focuses on understanding the neurobiology of fear, anxiety, aggression and pain, and the interrelationships between the circuitry that processes these emotions.

Our studies of fear are currently centered on the function of circuits in the amygdala, a medial temporal lobe structure that plays an important role in Pavlovian learned fear, a form of classical conditioning. We have identified genes that mark several subpopulations of neurons that form a dynamic microcircuit within the central nucleus of the amygdala. The function of this microcircuit in fear behavior is being dissected using optogenetic tools, such as channelrhodopsin, and pharmacogenetic tools, such as the ivermectin-gated glutamate sensitive chloride channel (GluCl) (Lerchener *et al*., 2007), together with acute slice electrophysiology and genetically-based anatomical tracing of synaptic pathways. Computational models for this circuit are being developed in collaboration with the Elowitz laboratory, in order to understand its function at a systems level and generate testable predictions.

Similar approaches are being used to understand the role of another brain region strongly implicated in stress and anxiety, the lateral septum (LS). There are conflicting data on whether the LS promotes or inhibits anxiety, due to a lack of information about its microcircuitry. We have gained a point-of-entry into this structure by focusing on neurons expressing the type 2 corticotrophin releasing hormone receptor (Crhr2), and are using a combination of transgenic and novel viral tools to understand the functional circuitry in which these neurons participate. In the case of aggression, we are focusing on circuits within the hypothalamus, an area that has extensive connectivity with both the LS and the amygdala, through which it receives input from the olfactory system (Choi *et al*., 2005). We are using chronic *in vivo* multi-unit recording to probe the relationship between neuronal activity and aggression, as well as mating, and are also employing genetically-based functional perturbations to understand how these two related social behaviors are processed by a common structure.

Pain has both a sensory and an affective component. We are using genetically targeted methods to probe the functional roles of different subpopulations of primary sensory neurons identified by the expression of Mrgprs, a family of orphan G protein-coupled receptors that we previously characterized (Dong *et al*., 2001). We are interested in the extent to which these receptors mark neurons specialized for particular types of painful stimuli, and if so how this specificity is conveyed to the brain.

Emotion circuits in Drosophila

The pioneering work of the late Seymour Benzer proved that the powerful genetics of *Drosophila* could be used to dissect the genetic underpinnings of many types of complex behaviors. Until recently, however, it was not clear whether this model system could also be applied to understanding the neurobiology of emotion and affect. We are taking two complementary approaches to determine the extent to which this is possible, and if so what we can learn from it. One approach is to dissect the neural circuitry

underlying behaviors that are analogous to defensive behaviors in higher organisms, such as avoidance (Suh *et al*., 2004, 2007), aggression (Wang *et al*., 2008) or immobility (Yorozu *et al*., 2009). The other is to model internal states or processes that are fundamental to many types of emotional responses, such as arousal, to ask for example whether arousal is unitary, or whether there are different types of behavior-specific arousal states (Lebestky *et al*., 2009). In both cases, we are developing novel behavioral assays, as well as machine vision-based approaches (Dankert *et al*., 2009) to automate the measurement of these behaviors (in collaboration with Pietro Perona, Allen E. Puckett Professor of Electrical Engineering), and are using molecular genetic-based tools to image and perturb neuronal activity in order to identify the specific circuits that mediate these behaviors.

Mouse Behavior

Selected publication

Liu, Q., Tang, Z., Surdenikova, L., Kim, S., Patel, K.N., Kim, A., Ru, F., Guan, Y., Weng, H.J., Geng, Y., *et al.* (2009) Sensory neuron-specific GPCR Mrgprs are itch receptors mediating chloroquine-induced pruritus. *Cell* **139**:1353-1365.

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265. Connectivity and function of lateral septal Crhr2+ neurons

T. Anthony

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 is of 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 exact function nor their synaptic connections 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 are yielding highly specific results about an anatomically and genetically defined neuronal population; such information is a prerequisite in the development of targeted anxiolytic drugs.

266. Genome-wide search for genes that regulate aggression of *Drosophila*

Kenta Asahina

Aggressive behaviors are prevalent among most animals including the fruit fly *Drosophila melanogaster*, a powerful genetic model for studying animal behavior. Although the impact that excessive human aggression has on society is clear, little is known about the molecular mechanisms and neuronal circuits that dictate aggressive behaviors. To obtain insight into such molecular machinery, we initiated a forward genetic screen in the fruit fly. Our goal is to isolate a collection of genes that normally suppress fly aggressive behaviors. Specifically, we are using RNA interference (RNAi) to reduce or eliminate the expression of individual genes in the nervous system. If a gene normally suppresses aggression, knock down of such a gene will confer flies with an unusually high level of aggressive behaviors. To facilitate the identification of hyperagressive phenotypes, we rear newly eclosed male flies as a group of 10~15 for 6 days. Such "group housed" flies are known to show greatly reduced levels of aggression compared to a male fly reared in isolation soon after eclosion. In a pilot screen, we tested ~150 genes for which the expression level has been shown to correlate with a fly's aggressiveness. By knocking down these genes specifically in neurons, we identified five genes that caused increased aggression (as determined by the number of lunges that occurred during a 20-minute period) when knocked down compared to the control strain. We plan to retest these five genes, and to test to what extent the elevated aggressive behavior is specific by observing the effect of knock down of these five genes on fly locomotion, courtship behaviors, and the circadian rhythm. We are also currently extending our screening to a wider range of genes expressed, or suggested to function in the nervous system.

267. Amygdala neural circuitry underlying mouse emotional behavior

Haijiang Cai, Wulf E. Haubensak, David J. Anderson

The amygdala neural circuitry plays a key role in the emotional behaviors that are critical for survival; however, with traditional lesion studies the cellular complexity makes it difficult to understand in detail how they regulate emotions. Here we use two novel strategies to study the specific neural circuitry of emotion. First, there are some molecular markers expressed only in subpopulations of amygdala nuclei. We take advantage of transgenic mice that express Cre recombinase in these marker-labeled neurons and manipulate the neural activity of these neuron populations by expressing Cre-dependent ChR2/NpHR or GluCl/IVM. For example, we have identified a genetic-marker(PKC-delta)-labeled subpopulation of CEl (lateral division of central amygdala) neurons, and demonstrated that they function as a circuit gating fear responses (see the abstract of Wulf Haubensak, *et al.*). Using optogenetics combined with electrophysiology, we mapped both local and long-range (cortical) functional inputs to these CEl neurons, as well as outputs from them. We found that the CEl neurons make inhibitory synapses onto projection neurons in the CEm (medial part of the central amygdaloid nucleus), which can be back-labeled from the PAG (periaqueductal gray), a brain region that mediates freezing behavior during the fear response. Interestingly we found the local inhibition within the CEl population is 10 times stronger than the inhibition of projection neurons in CEm. Second, different brain regions have different functions not only because they have different types of neurons but also because they are connected to different target brain regions. For example, we found that a projection from the insula cortical area is specifically located in the contralateral CEl, which gives us a strategy to manipulate a specific neural pathway or the activity of the subpopulation of insula neurons that project to contralateral CEl. The structure of the neural circuits and their functions characterized here will help us to understand better how the amygdala neural circuitry regulates emotional behaviors.

268. Fear control by inhibitory gating in the amygdala

Wulf Haubensak, Prabhat Kunwar, Haijiang Cai, David J. Anderson

Emotions are a central part of our experiences and guide most of our behaviors. Fear, the most basic and biomedically important emotion, for instance, is a reaction to stimuli associated with danger, and in turn evokes defensive behaviors necessary for survival. This process is governed by integrating sensory information in the lateral amygdala, and relaying this information via the central amygdala (CE) to brainstem motor and autonomic control centers.

However, because of the high degree of neuronal heterogeneity in the amygdala, conventional surgical and pharmacological manipulations could not resolve which, and how, individual neuronal circuits mediate these functions. Here, we have used novel combined viral and mouse genetic approaches to address this problem. We have used region-specific marker genes to genetically target subsets of amygdala neurons for pharmacogenetic silencing using an ivermectin-gated chloride channel, optogenetic activation using channelrhodopsin, and virus-based neuroanatomical tracing. With this strategy, we have identified a bistable neural microcircuit in CE that gates amygdala output. In the absence of fear stimuli, this circuit is active and inhibits fear responses by inhibiting CE output neurons. The presentation of fear stimuli, in contrast, switches this circuit to its inactive state, which, in turn, disinhibits CE output ultimately triggering a fear response.

Interestingly, this inhibitory gate is also the target of benzodiazepines, anxiolytics for treatment of panic and

anxiety attacks in humans. It is our hope that these findings provide entry points for understanding neuronal circuit mechanisms of both fear and emotion, as well as anxiety disorders and their pharmacological treatment.

269. Identification of neural circuits that promote aggression in *Drosophila melanogaster*

Eric D. Hoopfer[1&2], Gerald M. Rubin[2], David J. Anderson

Similar to most animals, *Drosophila melanogaster* exhibit an innate capacity for aggressive behavior that enables them to compete for food, mates, and territory. Males will engage in a set of stereotyped agonistic interactions until a dominance hierarchy is established. Aggressive behavior in vertebrates and invertebrates is strongly influenced by neuromodulatory systems, as well as environmental factors such as past social experience with conspecifics. However, the neural circuits that mediate aggressive behavior, and where and how in this circuitry factors that influence aggression act, remain largely unknown.

To identify neural circuits involved in aggressive behavior, we have developed a high-throughput, automated assay of *Drosophila* male social behavior that uses machine vision software (CADABRA; Dankert *et al*., 2009) to quantify aggressive and courtship interactions between pairs of males. Using this system, we are conducting a large-scale GAL4 screen to identify neurons that promote aggression when transiently activated using the temperature-sensitive *Drosophila* TRPA1 channel. We have identified several lines that show a robust, temperature-dependent increase in aggression. A detailed analysis of these phenotypes shows an enhancement in both the frequency of aggressive interactions and the escalation to higher-intensity aggressive behaviors. Lastly, we describe results of secondary behavioral assays aimed at assessing the specificity of the neuronal activation phenotype to aggression; determining the necessity of the neurons for aggression; and assessing how the activation of these circuits influences the expression of aggression in different social contexts.

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270. Monitoring and controlling neuromodulation during behaviors in *Drosophila melanogaster*

Hidehiko Inagaki

Neuromodulators regulate the outputs of neural circuits and allow animals to exhibit proper behaviors under various conditions. In order to understand how neuromodulators control behaviors, it is critical to identify the subsets of neurons modulated under different behavioral states. Despite its importance, currently there is no method to visualize neuromodulation *in vivo*. Here I developed a new tool to label the neurons modulated by neuromodulator, and applied it to study the regulation of behavioral states in the fruit fly *Drosophila melanogaster*. In preliminary studies, I applied the Tango assay, a system to induce reporter gene expression in response to activation of G-protein-coupled receptors (GPCRs), to neuromodulator receptors of fruit flies. In both cell culture and in the brains of transgenic flies, this system activated reporter gene expression in a neuromodulator-specific and dose-dependent manner. Since this system works well as a sensor for neuromodulator in fly brains, I tested what kinds of neurons are modulated in various stress states of flies. With this experiment, I found that specific neurons are modulated under hunger states, which is critical for the hunger-induced changes in flies. In future studies, I will improve the signal to noise ratio of the system to permit driving neural effectors in the modulated neurons, which will help us determine whether the modulated neurons are necessary or sufficient for the behavioral states.

271. Genetic dissection of amygdala neuronal circuitry for fear and anxiety in mice

Prabhat S. Kunwar

An animal's survival depends on its capacity to identify which sensory stimuli are dangerous to its existence, and its ability to produce the appropriate responses to avoid or combat harmful stimuli. The neural circuitry that controls fear, which can be learned or innate, and anxiety govern these critical judgments. In human, dysfunctions of this circuitry are thought to result in different diseases such as chronic anxiety, PTSD, depression, and autism.

The brain region that is linked to these diseases, and which plays a crucial role in regulating fear and anxiety, is the amygdala, an almond-shaped structure located in the medial temporal lobe of the forebrain. Precisely identifying the structure and function of the neural circuit located in the amygdala is essential for understanding how fear and anxiety occur in the normal brain, what goes wrong in the amygdala of affected people, and how appropriate treatments can be developed against fear disorders. Out of its various nuclei, the central amygdala (CeA) plays a central role in these fear behaviors.

My goal is to precisely determine the roles of different neuronal subpopulations in the CeA and to identify the neural circuits that control these behaviors and disorders. In these experiments, I am using molecular genetic tools of neuronal silencing and activation to modulate electrical activities of these neurons to analyze their effects on the behaviors in mice. Furthermore, I am using the genetic methods of *trans*-synaptic tracers to determine the anatomical and functional relationship of CeA neuronal subpopulations, in order to achieve a circuit-level explanation for the behavioral phenotypes that are caused by *in vivo* functional perturbations of these subpopulations.

272. The role of Mrgprd-expressing neurons in itch

Hyosang Lee, Li-Ching Lo, David Anderson

Itch, one the most distressing of dermatological symptoms, is associated with numerous skin diseases, such as atopic dermatitis, as well as with extracutaneous disorders. Intense scratching causes skin damage, which further increases itchiness. Unfortunately, currently available itch medications are ineffective in many chronic itch conditions and often cause undesirable side effects. Despite its clinical importance, the etiology and neurobiology of itch are not well understood. Previous studies have demonstrated that a small number of primary sensory neurons in the dorsal root ganglia (DRG) are involved in itching. However, the identity of those neurons has not been clearly defined. We hypothesize that Mrgprd-expressing DRG neurons play an important role in itching because 1) Mrgprd is specifically expressed in DRG neurons that innervate only the skin, where itching sensation mainly occurs, and 2) the nerve endings of Mrgprd-expressing neurons in the skin terminate in the most superficial layer of the epidermis, where irritants and pruritogens can be readily detected. To test this hypothesis, we have been examining whether Mrgprd-expressing neurons are necessary for pruritogen-evoked scratching behavior in mice. In our preliminary experiments, mice ablated of Mrgprd-expressing neurons exhibited greatly reduced scratching responses to cutaneous injection of a protease-activated receptor agonist, SLIGRL, when compared to their wild-type littermates. In contrast, histamine-evoked scratching behavior was normal in mice ablated of Mrgprd-expressing neurons. These results suggest that Mrgprd-expressing neurons are selectively required for PAR-2-mediated itch.

273. Dissection of neural circuitry regulating pheromonal control of social behavior in *Drosophila*

Rod Lim, David J. Anderson

Aggression is an innate social behavior observed in many species that is important for access to potential mates or resources. Unchecked aggression and violence pose significant threats to our society and thus, their prevention is of major concern. Despite its importance, the neural mechanisms underlying aggression are poorly understood. *Drosophila melanogaster* is a powerful genetic model organism in which to identify genes and neural circuits underlying complex behaviors. Despite large anatomical differences between an insect brain and a vertebrate brain, the *Drosophila* model system has made many fundamental contributions to neuroscience, from genetic studies allowing the first cloning of potassium channels to recent behavioral studies, which have demonstrated that fruit flies have dedicated neural circuits mediating innate stress responses.

Recent studies in our lab have identified cVA as an aggression-promoting pheromone in *Drosophila*. cVA is detected by Or67d-expressing olfactory sensory neurons (Wang *et al*., 2010). Or67d+ OSNs are required for the ability of cVA to promote aggression and increasing electrical excitability of these neurons promotes aggression, suggesting that they have an important role in regulation of aggressive behavior. Or67d+ OSNs project to the DA1 glomerulus in the antennal lobe, the site of secondary olfactory sensory processing in the fly brain. Although olfaction has been well characterized at the levels of primary OSNs, little is known about the information processing, which takes place downstream in second- and third-order olfactory neurons. The DA1 glomerulus is Fru+, which presents an entry point for analysis of its role in aggression using genetic tools. Using intersectional genetic approaches, we have recently identified enhancer-trap lines, which label the DA1 glomerular projection neurons and putative downstream neurons in the lateral horn and the mushroom body. By using the various reagents available to electrically silence and activate these neurons, I will test whether they are required for the detection of cVA, and whether they are sufficient for promoting aggression.

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274. Differentiate reproduction and aggression pathways in rodents

Dayu Lin, David J. Anderson

Inter-male aggression and male-female mating are generally considered as distinct 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. Both behaviors rely heavily on olfactory and pheromonal input. Lesions of the medial hypothalamus and medial amygdala in rodents decrease the occurrences of both mating and fighting. Taken together, these data suggest that mating and agonistic behaviors may be subserved by a common network of steroid-hormone-sensitive limbic areas. It is unclear how these two heavily overlapped pathways produce two opposite behavior outcomes. One possibility is that two intermingled but distinct subpopulations of neurons mediate mating and fighting. Alternatively, the same population of neurons could mediate both mating and fighting through neuromodulation. The goal of this 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 permit rapid sampling across the entire brain. Our 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. Next, we adapted a method to compare c-fos expression induced during the two behaviors in the same animal. Our data suggest that, at least in some commonly activated regions, two largely distinctive sets of neurons are likely involved in mating and fighting, while the same behavior tends to recruit a stereotyped set of neurons. Given our poor

understanding of the aggression circuit in general, we decided to further investigate the functional roles of several hypothalamic regions in aggression based on our Fos results. Using reversible viral inactivation tools, we found that neurons in the ventrolateral region of the ventromedial hypothalamus and its surrounding regions are likely to be critical for aggression initiation. Finally, we used chronic recording in awake-behaving animals to understand the physiological responses of those neurons during various episodes of aggressive and sexual behaviors.

275. Tracing neural circuits with anterograde-specific herpes viruses

Liching Lo

Herpes viruses have been known to invade the nervous system of their host and spread in chains of synaptically connected neurons. As a result, it is possible to trace the entire hierarchically connected circuits within an animal. My study has utilized strain H129 of herpes simplex virus 1 (HSV-1), which spreads transneuronally in an anterograde direction. Strain H129 has been used to trace many sequential neural pathways, including visual and basal ganglia pathways. In order to define specific neural pathways, we have borrowed the idea from a strain of pseudorabies virus PRV-Ba2001 by exploiting the conditional HSV-1 replication. I have used the cre-lox site-specific recombination system to construct an HSV-1 strain that replicates only when it infects a cell expressing cre recombinase. Briefly, a gene cassette containing a codon-modified herpes thymidine kinase (HTK) is inserted into the HSV-1 thymidine kinase locus that only expresses thymidine kinase and td-tomato in the cells that express the cre recombinase. The new virus, named CDRC (cre-dependent replication competent), lacks the thymidine kinase gene and consequently cannot grow in non-mitotic, terminally differentiated neurons. When it infects a cre-expressing cell, site-specific recombination results in permanent expression of thymidine kinase and td-tomato. Therefore, after infecting a cre-expressing neuron, the resulting virus can replicate in any neuron (regardless of whether it expresses cre or not) that is synaptically connected to the cre-expressing neurons. By injecting CDRC into the brain or peripheral nervous tissues of transgenic mice that expressed cre under the control of specific neuronal promoters, only neurons that the cre-expressing neurons projected to are labeled. I am currently testing the CDRC viruses in various neural pathways including visual and olfactory systems using mice expressing cre in a subpopulation of the primary olfactory or retinal sensory neurons. In the injected mice, I have observed td-tomato expression in the regions of brain that has been reported to be primary, secondary, or distal connections to the retina or olfactory sensory neurons.

276. Control of neural stem-to-progenitor transition by CyclinD family members

Agnes Lukaszewicz

Dr. David Anderson's lab has been interested for several years in understanding the molecular control of neural fate specification during development, using motor neurons (MNs) as a model. MNs are derived from a specific progenitor domain: the pMN domain of the spinal cord. While carrying 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 homeostasis of the developing spinal cord.

We have shown that CyclinD1 and D2 are expressed in a distinct subset of precursors, CyclinD1 but not D2 expression being spatially and temporally correlated with neurogenesis. This led us to hypothesize that CyclinD1 may regulate neurogenesis, whereas CyclinD2 may regulate the maintenance of the NSC. CyclinD1 re-expression is indeed sufficient for glial-restricted progenitors to regain their neurogenic potential. Furthermore, loss- and gain-of-function experiments allowed us to demonstrate that CyclinD1 plays a key role in modulating neurogenesis, a neurogenic function that is structurally independent of its function as a cell cycle regulator. In order to decipher the molecular mechanism involved, we have focused on Notch signaling, a crucial pathway in neurogenesis. We have shown that it can be modulated by CyclinD expression in a very specific and meaningful way: CyclinD1 is necessary for Hes6 expression (a neurogenic effector of Notch pathway), whereas CyclinD2 is necessary for Hes5 expression (canonical effector of Notch pathway). Furthermore, we have demonstrated that Hes6 and Hes5 overexpression can rescue CyclinD1 and D2 loss of function, respectively, implying that the two types of proteins functionally interact. We believe this constitutes an unexpected result of great importance for the field. We are currently in the process of submitting those data for publication in *Nature Neuroscience.*

277. 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.

278. 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 A, B, 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 at what point they might intersect with 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 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 (Liu *et al*.). It is also suggested that they might mark the mouse 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 measurements of their activity *in vivo* using gene targeting technology.

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279. Regulation of male social behaviors by multiple chemosensory modules in *Drosophila melanogaster*

Liming Wang, David J. Anderson

As in many animal species, the male vinegar fly *Drosophila melanogaster* conducts social behaviors towards conspecifics in a gender-specific manner. Male flies perform predominantly courtship towards female flies. During male-male interactions, in contrast, high levels of male-male aggression and relatively low levels of male-male courtship are observed. Such gender-specific behavioral choices-of-males is believed to be critical for the survival and reproduction of *Drosophila* and other animal species. However, the exact sensory cues that determine the gender-specific behavioral choice of male flies, and the underlying neural basis of those choices, are not well understood. Here we provide direct evidence that long-chain cuticular hydrocarbons (CHs) of male flies determine male behavioral choice. Elimination of male CHs elicits male-male courtship while suppressing male-male aggression, which can be rescued by the presence of a predominant male CH molecule, 7-tricosene (7-T), but not 7-pentacosene (7-P), another representative male CH molecule. The behavioral effects of 7-T requires the gustatory receptor Gr32a, and activation of gustatory sensory neurons (GSNs) expressing Gr32a mimics the behavioral effects of 7-T. Moreover, elimination of the Gr32a gene or silencing Gr32a+ GSNs only recapitulates the reduced male-male aggression but not the increased male-male courtship phenotype observed in CH-eliminated males, suggesting the presence of redundant CH molecule(s) and GSNs that suppress male-male courtship. Notably, the increased male-male courtship in the absence of male CHs is not reflecting a default behavioral state, but rather is the result of the presence of courtship-promoting pheromone(s) that activates a group of olfactory sensory neurons (OSNs) expressing Or47b. Taken together, multiple classes of pheromones regulate male behavioral choice towards other males via multiple chemosensory modules. Our findings reveal the evolutionarily conserved regulations of social behaviors across animal species and highlight the remaining questions in the chemosensory regulations of male social behaviors in mammals.

280. 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.

Octopamine (OA), a biogenic monoamine structurally related to noradrenaline, is an important regulator of various kinds of behaviors in arthropods. Although there are some reports related to the roles of OA and OA-synthesizing neurons on aggressive behavior, the cellular targets on which OA acts to modulate aggressive behavior is still unclear. The goal of this project is to identify the targets of OA-synthesizing neurons responsible for aggressive behavior and to elucidate the function and neuronal-connectivity of the OA system, as well as how it modulates aggressive behavior at the circuitry level.

Firstly, in collaboration with the Rubin lab (Janelia Farm), we generated 37 driver lines expressing the GAL4 protein in octopamine-receptor-positive neurons under the control of various *cis*-regulatory regions of four octopamine receptors (Oamb, Oa2, Octβ2R, and Octβ3R). Using these Gal4 lines combined with Tubulin-Gal80ts, I expressed Kir2.1 to block the activity of those neurons in adult specific manner, and analyzed the function of each octopamine-receptor-positive neuron in male-male aggressive behavior. Among those 34 lines, Kir2.1 expression driven by 13 Gal4 lines resulted in suppression of aggressiveness. With these 13 GAL4 lines, I performed a neuronal activation assay by expressing NaChBac, a bacterially derived voltage-sensitive sodium channel, in those neurons that showed the phenotype in the silencing experiment and identified two GAL4 lines with the phenotype of an increased level of aggression. Then, I characterized the expression patterns of these two GAL4 lines by crossing them to fluorescent reporters. Currently, I am trying to see how OA modulates these neurons. Thus, we have been trying to functionally dissect *Drosophila* brains to understand how specific neuronal circuits control aggressive behavior.

281. Neuronal control of locomotor activity in the fruit fly

Allan M. Wong, Michael H. Dickinson, David J. Anderson

The fruit fly, when presented with various stimuli-whether it visual, olfactory, thermal, or mechanical responds with a change in behavior. These changes are accomplished through a coordinated set of movements by the appendages. We are interested in how and where in the brain these different stimuli are integrated and how the signals propagate to the muscles that move the appendages. In particular, we wish to understand if the neurons that transmit these signals from the brain to the thoracic ganglia carry multimodal commands from prior integrated sensory information or if they carry unimodal commands that represent discrete channels for sensory to motor action.

We have developed a tethered walking fly arena in which a tethered fly walks 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 also developed a preparation where we remove the cuticle from the fly head to permit two-photon imaging in the fly brain. We use channelrhodopsin, a light-activated ion channel, to activate neurons to elicit walking while the fly is on the ball. We use a pulled fiber optic cable to deliver light locally to different populations of neurons in the antenna and the brain to elicit turning in the tethered fly.

282. The organization and property of Johnston's organ neurons in *D. melanogaster*

Suzuko Yorozu, Allan Wong, Brian Fisher, Heiko Dankert, Maurice Kernan, Azusa Kamikouchi, Kei Ito, David J. Anderson

The fruit fly *Drosophila* responds to sound, wind, and gravity using a pair of sensory organs called Johnston's organ (JO) located in the antennae. There are approximately 480 JO neurons in each JO, and the cell bodies are organized in ring-like arrays. There are five classes of JO neurons, which project to five distinct regions of the antennal and mechanosensory motor center of the central brain, called zones A, B, C, D, and E. Using *in vivo* calcium response imaging and behavioral analysis, we have shown that zone A and B neurons are sensitive to sound, while zone C and E neurons are sensitive to wind. Importantly, sound and wind-sensitive neurons have distinct intrinsic response properties and are activated by different arista movements: The sound-sensitive neurons are phasically activated by vibration of aristae, and wind-sensitive neurons are tonically activated by unidirectional static deflection of aristae. Two types of wind-sensitive neurons, C and E neurons, are sensitive to wind blowing from the front and the rear, respectively. Interestingly, when E neurons are activated by wind from the front, C neurons are slightly inhibited. Conversely, when C neurons are activated by wind from the rear, E neurons are slightly inhibited. Our data suggest that these antagonistic activation patterns between C and E neurons are partly due to how cell bodies of these neurons are organized in the JO. Using photo-activatable GFP, we show that the cell bodies of C and E neurons are located at opposite ends of the JO. Further analysis of the relative location of cell bodies of sound and wind-sensitive neurons suggests that JO neurons are tonotopically organized. This tonotopic organization of JO neurons might also explain why two classes of sound sensitive-neurons show different frequency tuning.

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Summary: Research in the laboratory focuses on three areas: (1) biophysics of computation in nerve cells; (2) understanding selective visual attention and visual consciousness at the neuronal, behavioral and computational levels; and (3) develop biological-motivated vision algorithm that predict where people and animals will look in natural scenes (gaze prediction). For more details and 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. We are working on the *forward* problem of determining the local field potential (LFP) that results from the synaptic and electrical activity of 1,000 of neurons in the hippocampus. We are also considering the *inverse* problem, studying the extent to which the local field influences the timing of action potentials of neurons. Our experimental (recordings with up to 12 patch electrodes in layer 5 pyramidal neurons *in vitro*) and computational research clearly shows that gradients as low as 1 mV/mm in the extracellular field can significantly affect the synchrony of neurons. That is, not only synaptic but also ephaptic effects will influence the firing behavior of population of neurons. This work is done in collaboration with the laboratories of Gyuri Buzsaki at Rutgers/New Jersey and Henry Markram at the EPFL/Lausanne.

Our laboratory collaborates with the neurosurgeon and neuroscientist Itzhak Fried at UCLA, recording from 128 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. In on current work, we demonstrated that patients can selectively, rapidly, transiently and voluntarily control the firing behavior of selective neurons in the medial temporal lobe, enhancing the firing rate of some, while simultaneously suppressing the firing rate of others and leaving the bulk of neurons unaffected.

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 attentional selection (via saliency) and object recognition in the near-absence of focal attention, in visual search, in natural scene perception and as reward is modulated. Aspects of this work are done in collaboration with Antonio Rangel, Ralph Adolphs and Colin Camerer of Caltech and with Laurent Itti at USC. Together with Tomaso Poggio at MIT, we investigate neurobiologically plausible models of both, the ventral, object-recognition and the dorsal, attention visual streams. We continue to work on understanding how neurons in regions LIP and FEF - modulated by top-down information - implement a visual saliency map.

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. Using `Continuous Flash Suppression' (CFS) and other techniques we have invented to hide images from conscious perception, we can show that visual, selective attention is a distinct process from visual consciousness. In collaboration with Chris Adami of the Keck Graduate Institute and Giulio Tononi of the University of Wisconsin at Madison, we are studying Tononi's *Integrated Information Theory* of consciousness and apply the underlying complexity measures to both abstract, as well as concrete biological circuits and motifs.

283. The effect of spatially inhomogeneous extracellular electric fields on neurons

C.A. Anastassiou[1], S.M. Montgomery[2], M. Barahona[1], G. Buzsáki[2], C. Koch

The cooperative action of neurons and glia generates electrical fields, but their effect on individual neurons via ephaptic interactions is mostly unknown. Here, we analyze the impact of spatially inhomogeneous electric fields on the membrane potential, the induced membrane field, and the induced current source density of one-dimensional cables, as well as morphologically realistic neurons and discuss how the features of the extracellular field affect these quantities. We show through simulations that endogenous fields, associated with hippocampal theta and sharp waves, can greatly affect spike timing. These findings imply that local electric fields, generated by the cooperative action of brain cells, can influence the timing of neural activity.

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284. Transcranial electric stimulation entrains cortical neuronal populations in rats

S. Ozen[1], A. Sirota[1,2], M.A. Belluscio[1], C.A. Anastassiou[3],C. Koch, G. Buzsáki[1]

Low intensity electric fields have been suggested to affect the ongoing neuronal activity in both *in vitro* studies and in humans. However, the physiological mechanism of how weak electrical fields affect and interact with intact brain activity is not well understood. We performed *in vivo* extracellular and intracellular recordings from the neocortex and hippocampus of anaesthetized rats and extracellular recordings in behaving rats. Electric fields were generated by sinusoid patterns at slow frequency (0.8, 1.25 or 1.7 Hz) via electrodes placed on the surface of the skull or the dura. Transcranial electric stimulation (TES) reliably entrained neurons in widespread cortical areas, including the hippocampus. The percentage of TES phase-locked neurons increased with stimulus intensity and depended on the behavioral state of the animal. A TES-induced voltage gradient as low as 1 mV/mm at the recording site was sufficient to phase-bias neuronal spiking. The onset of TES-induced effects was instantaneous and persisted throughout stimulation without any sign of adaptation. Intracellular recordings showed that both spiking and subthreshold activity were under the combined influence of TES forced fields and network activity. We suggest that TES in chronic preparations may be used for experimental and therapeutic control of brain activity.

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285. Ephaptic coupling of cortical neurons

C.A. Anastassiou[1], R. Perin[2], H. Markram[2], C. Koch

The electrochemical processes that underlie neural function manifest themselves in ceaseless spatial and temporal fluctuations in the extracellular field. The extracellular potential recorded from the tip of an electrode is used to study neural interactions during various brain states. In general, it is regarded as an epiphenomenon of coordinated neural activity. Yet, as shown by the classical experiments by Jefferys and Haas, induced extracellular fields can feed back onto the electrical potential across the neuronal membrane via ephaptic coupling. The extent to which such ephaptic coupling alters the functioning of individual neurons and neural assemblies under physiological conditions remains unclear. To address this question at the level of individual neurons, we stimulated and recorded with up to twelve electrodes from rat cortical pyramidal neurons in slice. We show that extracellular fields of 0.5-5 mV/mm amplitude induce ephaptically-mediated changes in the somatic membrane potential of under 0.5 mV in amplitude under subthreshold conditions. Yet despite their small size, we demonstrate that these fields can strongly entrain action potential activity. Such entrainment is particularly effective for slow (less than 8 Hz) fluctuations of the extracellular field and leads to synchronized activity of nearby neurons in the absence of synaptic transmission. These findings demonstrate that endogenous brain activity, such as slow cortical activity or hippocampal theta and sharp waves, can impact neural population activity through ephaptic coupling under physiological conditions.

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286. Color Doppler jet area overestimates regurgitant volume when multiple jets are present

B.A. Lin[*], A.S. Forouhar[*], N.M. Pahlevan, C.A. Anastassiou, P.A. Grayburn, J.D. Thomas, M. Gharib

Color Doppler jet area (CDJA) is an important measure used to classify mitral regurgitation (MR) severity. We hypothesized that the presence and configuration of multiple regurgitant jets can alter CDJA quantification for fixed regurgitant volumes. This has relevance to MR assessment prior to treatment of diseased valves with multiple regurgitant orifices or after surgical or endovascular double orifice mitral valve repair

[*]*Equally contributing authors.*

287. On-line voluntary control of temporal lobe neurons by human thought

M. Cerf[1,2,], N. Thiruvengadam[3], F. Mormann[4], A. Kraskov[5], R. Quian Quiorga[6], C. Koch[†], I. Fried[7†]

Daily life continuously confronts us with an exuberance of external, sensory stimuli competing with a rich stream of internal deliberations, plans and ruminations. The brain must select one or more of these for further processing. How this competition is resolved across multiple sensory and cognitive regions is not known; nor is it clear how internal thoughts and attention regulate this competition. Recording from single neurons in patients implanted with intracranial electrodes for clinical reasons, we demonstrate that humans can regulate the activity of their neurons in the medial temporal lobe (MTL) to alter the outcome of the contest between external images and their internal representation. Subjects looked at a hybrid superposition of two images representing explicit concepts (familiar individuals, landmarks, objects, or animals) and had to enhance one image at the expense of the other, competing one, while the spiking activity of MTL neurons in different subregions and hemispheres was decoded in real-time to control the content of the hybrid. Subjects rapidly, reliably, and specifically regulated the firing rate of neurons deep inside their own brain, increasing the rate of some while simultaneously decreasing the rate of others. They did so by focusing onto one image that gradually became more visible on the computer screen in front of their eyes, thereby overriding sensory input. Based on the firing of these MTL neurons, the dynamics of the competition between visual images in the subject's mind was visualized via an external display.

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288. There's plenty of time at the bottom: the time spent before a saccade is generated is a complex interplay of competing saliency and decision

M. Mackay[*], M. Cerf, C. Koch

Various models have been proposed to explain decision making and the interplay between bottom-up and top-down mechanisms in driving our saccades rapidly to targets in the environment. In this work, we investigate this relationship using eye-tracking data from subjects viewing natural scenes to test bottom-up and top-down based attention allocation to high-level objects (faces and text). Our results suggest two distinct types of bottom-up saliency to objects within a visual scene that disappear within a few fixations, and modification of this saliency by top-down influences. We propose that these observations reflect a common cortical pathway that represents a utility signal that modulates the process of saccadic decision. In addition, we propose a subcortical pathway capable of generating rapid, accurate saccades to salient targets following cortical analysis of the visual scene.

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289. Reevaluating the sustained division of the attentional spotlight at high temporal resolution

J. Dubois, J. Macdonald, R. VanRullen

Presented at Vision Sciences Society (VSS), May 2010

Recent fMRI and EEG evidence suggests that attention can concurrently select multiple locations. Most studies, however, generally lack a sufficient temporal resolution to rule out a unique, rapidly switching spotlight sequentially sampling the target locations. For example, using a classic "frequency-tagging" technique Müller and colleagues demonstrated a concurrent EEG enhancement at the tagged frequencies of two disjoint target locations (Müller *et al.*, *Nature,* 2003). The temporal resolution of this technique is, however, limited to a quarter of a second and often more: a few stimulus cycles are necessary to determine EEG amplitude at the tagged frequency, and higher frequencies (being more sensitive to measurement noise) require even longer analysis windows. So, can the spotlight really be divided in a sustained manner? Or does attention switch faster than can be resolved using existing techniques?

The novel method we present here ("broadband frequency tagging") aims at resolving this fundamental question. Ten subjects monitored two patches of randomly varying luminance, to the left and right of fixation, for the appearance of a brief and subtle contrast decrement, over a period of 6.25 seconds. Both patches contained an equal amount of power in all frequency bands from 2 to 80Hz over the stimulus presentation period. But critically, at any given time the two stimuli had distinct (and independent) frequency signatures. Using time resolved EEG decomposition in the frequency domain, this broadband frequency tagging paradigm allows us to dynamically monitor the allocation of attention to the two targets. As all tagging frequencies (2-80Hz) can contribute to the decomposition and their respective contributions can be adjusted at will, this technique can be said to provide the best possible temporal resolution. Using this new method, we were able to reconsider the previous conclusion that multiple targets are monitored in parallel.

290. Integrated information and fitness increases with simulated evolution

J. Edlund, N. Chaumont, A. Hintze, C. Koch, G. Tononi, C. Adami

One of the hallmarks of evolved organisms is their ability to integrate disparate information sources to optimize their behavior. How this can be quantified and related to the functional complexity of the systems remains a challenging problem. We present several candidates that quantify system and information integration (as well as other more standard information-theoretical measures), and study their dependence on fitness as an artificial agent evolves to solve a navigation task in a simple environment. Our measures correlate with fitness (an independent arbiter of relative complexity) in different degrees across many independent evolutionary runs, implying that high fitness requires both information processing, as well as information integration.

To be presented at Neural Information Processing Systems, 2010.

291. Minimalist tethered rover for exploration of extreme planetary terrains

P. Abad-Manterola, J. Edlund, J.W. Burdick, A. Wu, T. Oliver, I. Nesnas, J. Cecava

Recent scientific findings suggest that some of the most interesting sites for future exploration of planetary surfaces lie in terrains that are currently inaccessible to conventional robotic rovers. To provide robust and flexible access to these terrains, we have been developing Axel, the robotic rover. Axel is a lightweight two-wheeled vehicle that can access steep terrains and negotiate relatively large obstacles because of its actively managed tether and novel wheel design. This article reviews the Axel system and focuses on those system components that affect Axel's steep terrain mobility. Experimental demonstrations of Axel on sloped and rocky terrains are presented.

292. Information-based metrics of integration, synergy, and holism in dynamical systems

V. Griffith, S.A. Fraker, C. Adami[*]*, C. Koch*

Despite many attempts, quantifying the complexity of neural systems remains hard. Balduzzi and Tononi help tackle this problem by introducing effective information, *ei*, which assesses how much a system of disjoint modules is informationally a single entity. In this paper we (1) show that *ei* conflates the related---yet distinct---concepts of system integration, synergy, and holism; introduce a novel measure quantifying each concept; and, (2) show how our three measures differ from average *ei* across sixteen emblematic examples.

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293. Inter-subject functional mapping using fMRI predicts stimulus identity

J. Harel

A vital assumption in systems neuroscience is that the receptive field properties of neurons are generally similar across individuals. Very little, however, is understood about how the physical configurations of such functionally similar neurons might correspond across brains, and what patterns govern such mappings if any. Here, we investigate inter-subject functional correspondence in visual cortex using the fMRI BOLD responses of two human subjects to the same natural image stimuli. We relax the assumption that anatomically similar regions should represent similar information, and furthermore that there should be a smooth functional mapping connecting each point in one brain to one in the other. Instead, we find for each point in one brain a population of points in the other which provide statistically significant explanation. We present a novel formalism for representing such a mapping, and provide a simple algorithm to efficiently find a statistically meaningful one. This approach enables us to identify which image in a candidate set of 160 images a subject was viewing with accuracy as high as 16% (chance 0.625%), based only on the activity of the other brain, and for each image, a completely separate training set. This is much stronger performance than traditional brain-reading fMRI models, and in-line with a new wave of multivariate analyses. Our approach can be applied to parametric neuroscience studies, such as neuroeconomics experiments, since it reveals what information about a stimulus condition is shared between two brains, and where.

294. Image signature: Simple vision using the sign function

X. Hou

In this paper, we demonstrate that a simple quantity, which we refer to as the Image Signature, can be used as the basis of a highly efficient visual saliency algorithm. The algorithm outperforms leading algorithms on benchmark datasets at just 10 to 16% the computational cost. We also present new data from a change blindness experiment in which we demonstrate that the image signature induces a distance measure between images which better accounts for perceptual differences in this context than the GIST descriptor. We conclude with a theoretical analysis of the connection between the image signature and foreground-background separation

Presented at NIPS, Dec 2009

295. Time course of target recognition in visual search

A. Kotowicz, U. Rutishauser, C. Koch

Visual search is a ubiquitous task of great importance: it allows us to quickly find the objects that we are looking for. During active search for an object (target), eye movements are made to different parts of the scene. Fixation locations are chosen based on a combination of information about the target and the visual input. At the

end of a successful search, the eyes typically fixate on the target. But does this imply that target identification occurs while looking at it? The duration of a typical fixation (~170 ms) and neuronal latencies of both the oculomotor system and the visual stream indicate that there might not be enough time to do so. Previous studies have suggested the following solution to this dilemma: the target is identified extrafoveally and this event will trigger a saccade towards the target location. However, this has not been experimentally verified. Here we test the hypothesis that subjects recognize the target before they look at it using a search display of oriented colored bars. Using a gaze-contingent real-time technique, we prematurely stopped search shortly after subjects fixated the target. Afterwards, we asked subjects to identify the target location. We find that subjects can identify the target location even when fixating on the target for less than 10 ms. Longer fixations on the target do not increase detection performance but increase confidence. In contrast, subjects cannot perform this task if they are not allowed to move their eyes. Thus, information about the target during conjunction search for colored-oriented bars can, in some circumstances, be acquired at least one fixation ahead of reaching the target. The final fixation serves to increase confidence rather then performance, illustrating a distinct role of the final fixation for the subjective judgment of confidence rather than accuracy

296. Single neuron representation of bias in primate value-based decision making

U. Maoz, S. Kim[1], U. Rutishauser[2], D. Lee[1], C. Koch

Two rhesus monkeys performed a two-alternative forced-choice task, between a smaller amount of juice at short or no delay and more juice at a longer delay, using eye movements (Kim *et al*., 2008). Single-cell activity was simultaneously recorded from their dorsolateral prefrontal cortex (DLPFC). The animals reliably selected the option with the higher subjective temporal discounted value.

We assigned each trial to one of four groups according to the consistency of the animals' choice given the small-reward and large-reward delays. The animals' response times increased with decreasing choice consistency, attesting to the increased decision difficulty. We hypothesized that cells whose pre-cue activity predicted the eventual saccade direction are those that code the decision bias of the animal. The animals knew neither the locations nor the corresponding delays of the large and small rewards before cue onset. So, for such bias cells, the difference in pre-cue activity between eventual left and right eye movement should decrease with the increase in consistency along the four groups. More than 25% (30 of 117) of the relevant recorded cells displayed such an activity pattern, as did the average activity over the entire cell population (bootstrapping p-value <0.005). The divergence between left and right eye movements for these bias cells was found to be highest before cue onset and then significantly decrease (one-sided paired t-test p-value<1.3^{-4}).

No significant correlations were found between the animals' behavior in successive trials (binomial test p-value=0.68), nor between the present choice and more complex behavioral history patterns (binomial test p-value=0.46). Lastly, a simple 5-component circuit model (incorporating a winner-take-all network with pointer-neurons biasing) reproduced the described neural activity and made further predictions. We thus, suggest that internal-state-based pre-cue DLPFC activity is incorporated into the deliberative decision making process with the bias's influence increasing with the similarity of the temporally discounted values of the two options.

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297. Are fast choices driven by value or visual saliency of the options?

M. Milosavljevic, V. Navalpakkam, C. Koch, A. Rangel

Imagine that you are in a hurry and have a few seconds to choose a food item. The available items vary both in subjective value - how much you like each – and in their visual saliency – brightness. How do the two factors affect your choice? Here, subjects made choices between pairs of food items with different subjective values while we manipulated visual saliency by changing the brightness of the options. The results indicate that visual saliency affects choices when the value of the two items is similar, but that value wins over when one of the options is highly preferred.

Presented at the Society for Judgment and Decision-Making Conference, November 2009

298. The nature of salience in games: A biological perspective

A. Smith, M. Milosavljevic, C. Koch, C. Camerer

The framing of a game may influence play through the saliency of decision labels. Definitions of saliency in game theory are typically informal: salient labels 'stick out' or 'suggest themselves.' We suggest that low-level perceptual features such as colors, brightness, orientation, size, or motion may influence strategic behavior in games through neural mechanisms that direct attention to visually salient stimuli. We hypothesize that a biologically inspired low-level model of visual attention, combined with a cognitive hierarchy model of strategic thinking, can predict changes in strategic behavior resulting from different frames. We study this hypothesis by varying the visual presentation of matching and coordination games in laboratory settings.

Presented at the BQGT Conference, May 2010

299. Optimal reward harvesting in complex perceptual environments

V. Navalpakkam, C. Koch, A. Rangel, P. Perona

The ability to choose rapidly among multiple targets embedded in a complex perceptual environment is key to survival. Targets may differ both in their reward value, as well as in their low-level perceptual properties (e.g., visual saliency). Previous studies investigated separately the impact of either value or saliency on choice, thus, it is not known how the brain combines these two variables during decision-making. We addressed this question with three experiments in which human subjects attempted to maximize their monetary earnings by rapidly choosing items from a brief display. Each display contained several worthless items (distractors), as well as two targets, whose value and saliency were varied systematically. We compared the behavioral data to the predictions of three computational models which assume that: (1) subjects seek the most valuable item in the display; (2) subjects seek the most easily detectable item; and, (3) subjects behave as an ideal Bayesian observer who combines both factors to maximize expected reward within each trial. We find that, regardless of the type of motor response used to express the choices, decisions are influenced by both value and feature-contrast in a way that is consistent with the ideal Bayesian observer, even when the targets' feature-contrast is varied unpredictably between trials. This suggests that individuals are able to harvest rewards optimally and dynamically under time pressure while seeking multiple targets embedded in perceptual clutter.

300. Homo economicus in visual search

V. Navalpakkam, C. Koch, P. Perona

How do reward outcomes affect early visual performance? Previous studies found a suboptimal influence, but they ignored the non-linearity in how subjects perceived the reward outcomes. In contrast, we find that when the non-linearity is accounted for, humans behave optimally and maximize expected reward. Our subjects were asked to detect the presence of a familiar target object in a cluttered scene. They were rewarded according to their performance. We systematically varied the target frequency and the reward/penalty policy for detecting/missing the targets. We find that: 1) Decreasing the target frequency will decrease the detection rates, in accordance with the literature; 2) Contrary to previous studies, increasing the target detection rewards will compensate for target rarity and restore detection performance; 3) A quantitative model based on reward-maximization accurately predicts human detection behavior in all target frequency and reward conditions; thus, reward schemes can be designed to obtain desired detection rates for rare targets; and, 4) Subjects quickly learn the optimal decision strategy; we propose a neurally plausible model that exhibits the same properties. Potential applications include designing reward schemes to improve detection of life-critical, rare targets (e.g., cancers in medical images).

301. Optimal visual search under uncertainty with probabilistic population codes

W.J. Ma, V. Navalpakkam, J. Beck, R. van de Berg, A. Pouget

The ability to search efficiently for a target in a cluttered environment is one of the most remarkable functions of the nervous system. This task is difficult in natural environments in part because the reliability of sensory information can vary greatly across space and time, and is typically *a priori* unknown to the observer. In contrast, standard visual search experiments use stimuli that all come with equal and known reliability. To test whether human subjects indeed perform optimal visual search in more realistic situations, we randomly assigned high or low reliability to each item on a trial-by-trial basis in a target detection task. We found that humans are near optimal, regardless of whether distractors were identical or drawn from a uniform distribution. We also present a neural network implementation of optimal visual search, based on the formalism of probabilistic population coding. The network matches human performance without requiring reliability to be estimated separately from stimulus encoding.

302. Expected value has a strong effect on naturalistic search

R. Pederisini, V. Navalpakkam, T. Horowitz, J. Wolfe

Previous studies demonstrated a strong prevalence effect in naturalistic search, i.e., rare targets often missed in simulated airline baggage screening tasks. Here, we investigate the effect of reward payoffs in such complex scenarios. We ask three questions: 1) Do reward incentives have a significant effect on visual search performance? 2) If so, can they can they counteract the effects of target prevalence? 3) Do observers performing visual search behave as value maximizers, or do they maximize search accuracy instead? In three experiments, observers performed a simulated x-ray baggage screening task, searching in synthetic x-ray images of luggage for weapons among other objects. We employed various payoff matrices, which affected observers' actual monetary reward. Experiment 1 demonstrated that payoff matrices can significantly affect search behavior. Experiment 2 showed that this is true even at low prevalence, when targets appeared on only 10% of trials. Finally, Experiment 3 used payoff matrices designed to distinguish between accuracy maximization and value maximization. When compelled to choose between accuracy and value, observers maximized value.

303. Why candy wrappers should be red and bright: The effects of irrelevant perceptual features on choice

M. Milosavljevic, V. Navalpakkam, C. Koch, A. Rangel

Are realistic choices primarily driven by reward value, or do visual factors, unrelated to value, also matter? We asked hungry volunteers to make rapid choices between food items that varied in value (preference/liking) and visual properties like saliency. We found that, controlling for value, objects with more salient visual properties (i.e., brighter items) are more likely to be chosen. Furthermore, the magnitude of this effect decreases with the amount of time that subjects have to process the stimuli, and increases under conditions of cognitive load and when the stimuli are of similar value. Thus, the interaction between value and visual saliency varies with time, with faster choices being driven by saliency, and slower choices by value.

304. Topographic organization of V1 projections through the corpus callosum in humans

M. Saenz, I. Fine

The visual cortex in each hemisphere is linked to the opposite hemisphere by axonal projections that pass through the splenium of the corpus callosum. Visual-callosal connections in humans and macaques are found along the V1/V2 border where the vertical meridian is represented. Here we identify the topography of V1 vertical midline projections through the splenium within six human subjects with normal vision using diffusion-weighted MR imaging and probabilistic diffusion tractography. Tractography seed points within the splenium were classified according to their estimated connectivity profiles to topographic subregions of V1, as defined by functional retinotopic mapping. First, we report a ventral-dorsal mapping within the splenium with fibers from ventral V1 (representing the upper visual field) projecting to the inferior-anterior corner of the splenium and fibers from dorsal V1 (representing the lower visual field) projecting to the superior-posterior end. Second, we also report an eccentricity gradient of projections from foveal-to-peripheral V1 subregions running in the anterior-superior to posterior-inferior direction, orthogonal to the dorsal-ventral mapping. These results confirm and add to a previous diffusion MRI study (Dougherty *et al*., 2005) that identified a dorsal/ventral mapping of human splenial fibers. These findings yield a more detailed view of the structural organization of the splenium than previously reported and offer new opportunities to study structural plasticity in the visual system.

305. Realistic models of extracellular potentials: From single units to LFPs

E.W. Schomburg, C.A. Anastassiou, C. Koch

Recordings from extracellular electrodes implanted in the brain are ubiquitous in the neurosciences, since they convey information about distributed input and population activity. High frequency components (> 300 Hz) are associated with neuron action potentials. Low frequency components (< 300 Hz), termed the local field potential (LFP), mostly reflect the collective synaptic input to the region around the electrode. Previous work in our lab has focused on modeling extracellular signatures of action potentials (Gold *et al*., *J. Neurophysiol*., 2006). However, the precise mechanisms and the spatial extent of the population contributing to LFP generation are not well understood. To improve our ability to interpret extracellular electrode recordings in the brain, we simulate populations of neurons and generate synthetic electric potentials from the resulting membrane currents. Our model system is the CA1 region of the rat hippocampus, an oft-studied structure ideally suited for the occurrence of large electric potentials due to its laminated architecture with well-aligned principle cells receiving temporally coordinated synaptic input, distributed in a spatially polarized manner along the cells. Using detailed neuron models, we are considering an array of processes that contribute to the currents generating these field potentials and analyzing the relative importance of their contribution to the LFP. The accompanying figure shows electric potential traces (with respect to a reference at infinity) from a line of seven electrodes in the middle of a sheath of pyramidal cells (95% of which remain subthreshold throughout the simulation) with their apical axes aligned. An example cell with marked synapses is shown on the left, and the color map on which the electrode traces are overlaid is the current source density (CSD) along the electrode shank, with increasing time going to the right (as in the electrode traces).

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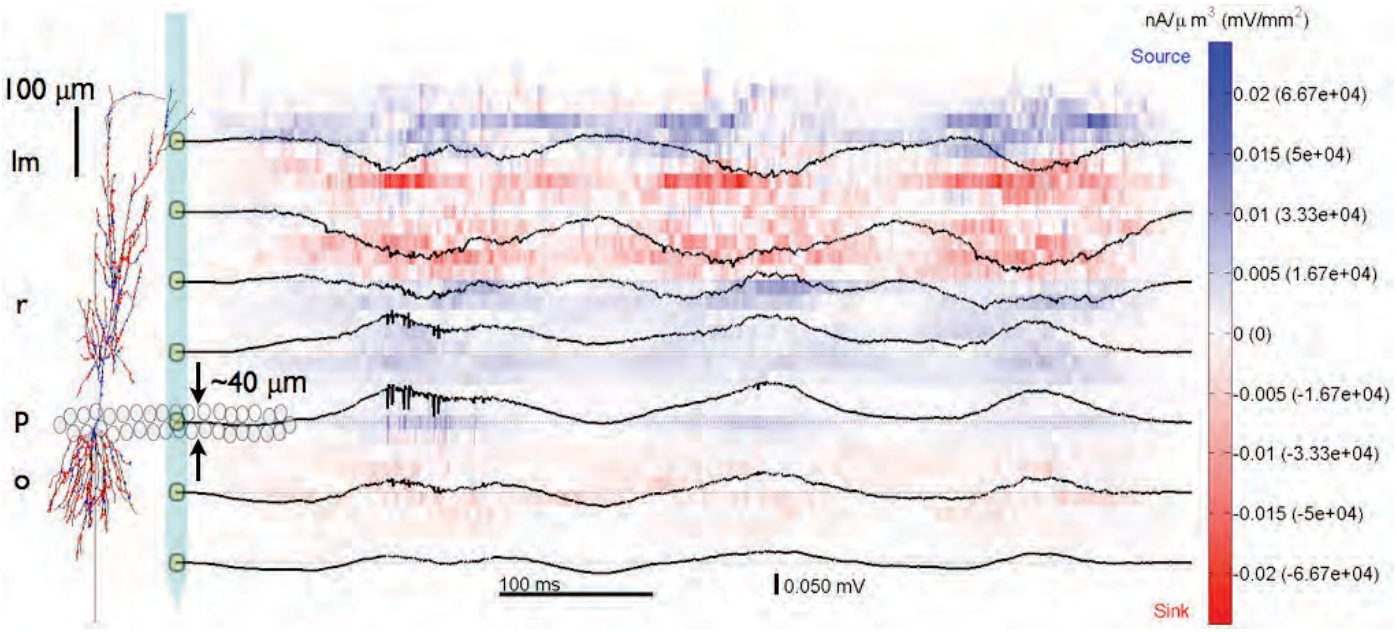

Figure 1: Simulated electric potential traces at seven locations on a linear electrode array overlay the current source density along the electrode shank. Time increases from left to right for both measures. The population consists of 2010 morphologically identical pyramidal cells with four distinct synaptic input patterns, uniformly distributed in a 0.8 mm diameter sheath and normally distributed in time (15 ms standard deviation). The synaptic input is periodic at 5 Hz, though not identical across periods. Two of the patterns result in action potentials during some input cycles (5% of the cells), whereas two remain subthreshold for the duration shown here. Sources arise where ionic current flows into a cell, and sinks appear where current exits a cell. On neural timescales, the total current crossing a cell's membrane is approximately zero, but its spatial segregation generates the potential differences measured by the electrodes.

306. Opposing visual saliency computations: mechanisms, constraints, and the effect of feedback

A. Soltani, C. Koch

The primate's visual system continuously selects spatial proscribed regions, features or objects for further processing. These selection mechanisms - collectively termed selective visual attention - are guided by intrinsic, bottom-up and by task-dependent, top-down signals. While much psychophysical research has shown that overt and covert attention is partially allocated based on saliency-driven exogenous signals, it is unclear how this is accomplished at the neural level. Electrophysiological experiments in monkeys point to the gradual emergence of saliency signals when ascending the dorsal visual stream and to the influence of top-down attention on these signals. In order to elucidate the neural mechanisms underlying these observations, we construct a biologically-plausible network of spiking neurons to simulate the formation of saliency signals in different cortical areas. We find that saliency signals are rapidly generated through lateral excitation and inhibition in successive layers of neural populations selective to a single feature. These signals can be improved by feedback from a higher cortical area that represents a saliency map. In addition, we show how top-down attention can affect the saliency signals by disrupting this feedback through its action on the saliency map. While we find that saliency computations require dominant slow NMDA currents, the signal rapidly emerges from successive regions of the network. In conclusion, using a detailed spiking network model we find biophysical mechanisms and limitations of saliency computations that can be tested experimentally.

307. Opposing effects of attention and consciousness on afterimages

J.J.A van Boxtel, N. Tsuchiya, C. Koch

The brain's ability to handle sensory information is influenced by both selective attention and consciousness. There is no consensus on the exact relationship between these two processes and whether or not they are distinct. So far, no experiment simultaneously manipulated both. We carried out the first full factorial 2x2 study of the simultaneous influences of attention and consciousness (as assayed by visibility) on perception, correcting for possible concurrent changes in attention and consciousness. We investigated the duration of afterimages for all four combinations of high versus low attention and visible versus invisible grating. We demonstrate that selective attention and visual consciousness have opposite effects: paying attention to the grating decreases the duration of its afterimage, while consciously seeing the grating increases the afterimage duration. These data provide clear evidence for distinctive influences of selective attention and consciousness on visual perception.

308. Multisensory congruency as a mechanism for attentional control over perceptual selection

R. van Ee, J.J.A. van Boxtel, A. Parker, D. Alais

The neural mechanisms underlying attentional selection of competing neural signals for awareness remains an unresolved issue. We studied attentional selection, employing perceptually ambiguous stimuli in a novel multisensory paradigm that combined competing auditory and competing visual stimuli. We demonstrate that the ability to select, and attentively hold, one of the competing alternatives in either sensory modality is greatly enhanced when there is a matching crossmodal stimulus. Intriguingly, this multimodal enhancement of attentional selection seems to require a conscious act of attention, as passively experiencing the multisensory stimuli did not enhance control over the stimulus. We also demonstrate that congruent auditory or tactile information, and combined auditory-tactile information, aids attentional control over competing visual stimuli, and visa versa. Our data suggest a functional role for recently found neurons that combine voluntarily initiated attentional functions across sensory modalities. We argue that these units provide a mechanism for structuring multisensory inputs that are then used to selectively modulate early (unimodal) cortical processing, boosting the gain of task-relevant features for willful control over perceptual awareness.

309. Attending to auditory signals slows visual alternations in binocular rivalry

D. Alais, J.J.A. van Boxtel, A. Parker, R. van Ee

A previous study has shown that diverting attention from binocular rivalry to a visual distractor task results in a slowing of rivalry alternation rate between simple orthogonal orientations. Here, we investigate whether the slowing of visual perceptual alternations will occur when attention is diverted to an auditory distractor task, and we extend the investigation by testing this for two kinds of binocular rivalry stimuli and for the Necker cube. Our results show that doing the auditory attention task does indeed slow visual perceptual alternations, that the slowing effect is a graded function of attentional load, and that the attentional slowing effect is less pronounced for grating rivalry than for house/face rivalry and for the Necker cube. These results are explained in terms of supramodal attentional resources modulating a high-level interpretative process in perceptual ambiguity, together with a role for feedback to early visual processes in the case of binocular rivalry.

310. Visual rivalry without spatial conflict

J J.A. van Boxtel, C. Koch

When multiple perceptual solutions are approximately equally likely given the context, perception may alternate between different solutions, i.e., visual rivalry. Visual rivalry has been studied for centuries with spatially defined conflict, i.e., spatially overlapping percepts, whether they are dichoptic gratings, ambiguously-moving dots, or foreground-background reversals. Thus, it seems that visual rivalry requires spatial conflict. Can visual rivalry occur without spatial conflict? We show that visual rivalry occurs even in the absence of spatial or temporal conflict when we place an ambiguous motion quartet in an object-centered, i.e., non-retinal, and reference frame. Conversely, a motion quartet that is displaced within an object-centered reference frame does not induce rivalry even in the presence of retinal conflict. Thus, visual conflict resolution takes place continuously in non-retinal reference frames, subserving object-based perception, and is able to overrule retina-based processing.

311. Consciousness and attention: On sufficiency and necessity

J.J.A. van Boxtel, N. Tsuchiya, C. Koch

Recent research has slowly corroded a belief that selective attention and consciousness are so tightly entangled that they cannot be individually examined. In this review we summarize psychophysical and neurophysiological evidence for a dissociation between top-down attention and consciousness. The evidence includes recent findings that show subjects can attend to perceptually invisible objects. More contentious is the finding that subjects can become conscious of an isolated object, or the gist of the scene in the near absence of top-down attention. We also cover the recent flurry of studies that utilized independent manipulation of attention and consciousness. These studies have shown paradoxical effects of attention and cases where top-down attention and consciousness have opposing effects. Neuroimaging studies with EEG, MEG and fMRI are now uncovering the distinct neuronal correlates of selective attention and consciousness in dissociative paradigms. Separating the effects of selective visual attention from those of visual consciousness is of paramount importance to untangle the neural substrates of consciousness from those for attention.

312. Correlated effects of attention and awareness on contrast threshold elevation but not on afterimage formation

J.W. Brascamp, J.J.A. van Boxtel, T. Knapen, R. Blake

In the debate about the relation between attention and awareness a curious observation is that the two phenomena have opposite effects on afterimage formation: unperceived inducing images leave a weaker afterimage whereas unattended inducing images leave a stronger afterimage. This qualitative difference stands out among other findings that generally show attention and awareness to be similar, albeit not identical. Our starting point is the observation that, beside an afterimage, inducing images also cause contrast threshold elevation. Indeed, this threshold elevation impairs visibility of the afterimages themselves. This renders inconclusive existing reports of opposite effects of attention and awareness on subsequent afterimage perception: a fainter afterimage could either indicate lessened afterimage formation or augmented threshold elevation. We present a new psychophysical

method to tease apart these two factors and thereby clarify the effects of attention and awareness on afterimage formation. Using this method, which centers on nulling the afterimage using a physical image, we demonstrate that attention and awareness have similar effects on afterimage formation, and also on threshold elevation. Both are augmented when the inducing image is attended, as well as when the inducing image is perceived. The impression of opposite effects of attention and awareness only arises when afterimage strength and threshold elevation are not distinguished, such as in measures of afterimage visibility. In addition, we show that inter-observer differences in the effects of attention and awareness on threshold elevation are correlated. Inter-observer differences in the effects on afterimage formation, however, are not. Our results indicate that attention and awareness are qualitatively similar in their effects on afterimage formation and threshold elevation, and that this similarity is particularly pronounced at the level of threshold elevation.

313. Attention, surface and boundary signals in the perception of afterimages

J.J.A. van Boxtel, C. Koch

Currently, it is believed that a stimulus is visible as long as it has a surface signal (which is the signal that is perceived) surrounded by a strong-enough boundary signal (which itself is not visible). Without a strong boundary signal the surface signal dissipates and the stimulus fades from awareness.

One problem with this hypothesis is that the border signal is experimentally (and conceptually) confounded with the visibility of the stimulus. Because the boundary signal has never been experimentally separated from the visibility of the stimulus, the core of the hypothesis has not been tested. To circumvent this problem, we simply physically present a ring on the screen surrounding the afterimage. This ring will provide a strong boundary signal. We show that the duration of the afterimage is lengthened by about 50% with a ring that exactly encompasses the afterimage. Rings of equal or smaller size than the afterimage increase afterimage duration relative to a condition without a ring, while boundaries larger than the afterimage do not increase afterimage duration. We find furthermore that maximum modulation occurs for intermediate contrasts of the ring, making attentional capture (by large luminance changes) an unlikely cause of the effect. Thirdly, placing a ring around the position of an already faded afterimage, revives the afterimage. Finally, we modulated the amount of attention paid to the adapting stimulus. We found that both boundary and surface signals were more adapted with increased amounts of attention. Interestingly, increased adaptation of the boundary signal led to decreased afterimage durations, while increased adaptation to the surface signals led to increased afterimage durations. Our data show that boundary signals (i.e., the ring) are crucial in the determination of afterimage perception. We also show that attention acts on both surface and boundary information, and that it has the capability to both increase and decrease afterimage duration.

314. Making better predictions where people look in photos: Learning feature integration

Q. Zhao, C. Koch

Inspired by the primate visual system, computational saliency models decompose the visual input into a set of feature maps across spatial scales. In the standard approach, the feature maps of the pre-specified channels are summed to yield the final saliency map. Although such a linear summation scheme has some psychophysical support and is simple to apply, there is no relevant neurophysiological evidence for such a linear scheme. Furthermore, some psychophysical arguments have been raised against linear integration strategies.

We study feature integration strategies of computational saliency models using eye movement data. Based on the conventional linear summation approach, we first improve the linear method by learning a set of optimal feature weights using the constraint linear least square algorithm. Humans depend more on certain features than others in deciding where to look at, and the predictivity of the saliency model improves significantly by addressing such differences by learning optimal weights. Using eye movement data from three public eye tracking datasets (Bruce and Tsotsos, 2009, Cerf, *et al.*, 2009, Judd *et al.*, 2009), we found that among the four bottom-up features, face is the most important one in discriminating fixations and non-fixations, followed by orientation, color, and intensity.

We further propose an AdaBoost based algorithm that provides a principled computational framework for feature selection and integration. It makes no assumption of linear superposition or equal weights of features. Indeed, quantitative evaluations on the three datasets explicitly demonstrate that certain types of nonlinear combination consistently outperform linear combination, even with optimal weights. This indicates that linear approximation is still insufficient to model the complex way that humans use to integrate different features, and raises the question of the extent to which the primate brain takes advantages of such nonlinear integration strategies. Biological neurons are highly nonlinear devices. Thus, implementing the type of nonlinearities inherent in AdaBoost is not particular problematic for the brain. Future psychophysical and neurophysiological research will be needed to untangle this question.

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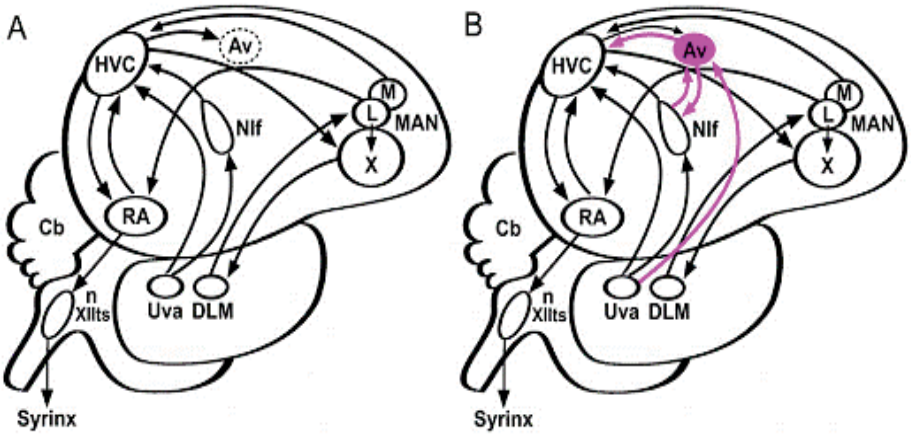
Summary: All birds use sounds to communicate with each other. The so-called songbird is particularly interesting to us, because young birds imitate song either from their fathers or other males in the vicinity. Song learning consists of two stages, memorizing a song during youth and reproducing it in adulthood. We would like to know where in the brain and in what form tutor song is stored. We know the chain of discrete brain areas that control song. These areas are unique to bird species that learn song and the areas are collectively referred to as the song control system. However, our own follow-up studies have revealed several new connections between areas that were thought to form a simple chain. Thus, our findings make the song control system much more complex than it was originally reported (Fig. below). It shows the old figure and B shows the new figure based on our data. The red lines indicate the newly found connections.



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Summary: Our lab studies ion channels that respond to the neurotransmitters acetylcholine, serotonin, GABA, glycine, and (among invertebrates) glutamate. These are termed "Cys-loop receptors." At the most fundamental level, with Professor Dennis Dougherty's group in Caltech's Division of Chemistry and Chemical Engineering and Professor Sarah Lummis of Cambridge University, we apply new types of chemistry to understand how Cys-loop receptors transduce the binding of agonists into the opening of the channels.

We are describing the neural events that occur when an animal is chronically exposed to nicotine. We hypothesize that this set of responses underlies the pathophysiology of nicotine addiction, the world's largest preventable cause of death.

There is no medical justification for the use of smoked tobacco. However, the organism's responses to chronic nicotine also underlie the benefits of two inadvertent therapeutic effects of smoking: the inverse correlation between a person's history of smoking and his/her susceptibility to Parkinson's disease, and the preventive effect of nicotine in autosomal dominant nocturnal frontal-lobe epilepsy.

We are studying these complex neural processes at several appropriate levels: the genes, the receptor proteins, the effects on neurons, the organization of neurons in circuits, the resulting behavior of animals, and even neural events in humans. Several lab members are describing the molecular/biophysical aspects of SePhaChARNS, for "selective pharmacological chaperoning of acetylcholine receptor number and stoichiometry." We hypothesize that SePhaChARNS is a thermodynamically driven process leading to the classical observation that chronic exposure to nicotine causes "upregulation" of nicotinic receptors. Other proteins modify SePhaChARNS; and lab members are studying two of these: the modulatory protein lynx, and the auxiliary nicotinic subunit $\alpha 5$. If the hypothesis is proven, SePhaChARNS is the molecular mechanism that shapes an animal's response to chronic exposure to nicotine. We are now producing subcellular movies depicting the first 24 hours of nicotine addiction—thought to be the most crucial-stage in the process, especially for adolescents. These images display the spread of newly chaperoned, fluorescent receptors as they travel from the endoplasmic reticulum to the cell membrane.

Other lab members are generating mice with genetically modified nicotinic receptors—gain of function, not knockouts. Some mice have a hypersensitive subunit; in such mice, responses to nicotine represent selective excitation of receptors containing that subunit. Other mice have a fluorescent subunit, so that we can quantify and localize upregulation of receptors containing that subunit.

We are also engineering Cys-loop receptors in order to manipulate neurons. Now we are optimizing suitably mutated *C. elegans* GluCl receptor channels to have strong responses to minuscule concentrations of the anthelmintic ivermectin and its derivatives. In collaboration with Professor David Anderson's group, we have introduced these channels into mouse neurons. When the animal is injected with ivermectin, the neurons can be selectively silenced—and eventually, we hope, selectively activated. If we can improve the system, there are important applications for both research and therapy.

Several of our projects lead naturally to drug discovery procedures. We have a drug discovery collaboration with Michael Marks and his group at the University of Colorado, Boulder; and with Targacept, Inc. We also collaborate with Robert Freedman and his colleagues at the University of Colorado, Denver, to generate scientific knowledge and therapies around the heavy smoking by schizophrenics.

We also have interests in new techniques at the intersection of biophysics, single-molecule imaging, chemistry, mouse genetics, and neuroscience. We're delighted to host visitors in our lab on the third floor of the Kerckhoff Laboratory.

315. The nicotinic pharmacophore: The pyridine N of nicotine and carbonyl of ACh hydrogen bond across a subunit interface to a backbone NH

Angela P. Blum[*], Henry A. Lester, Dennis A. Dougherty[*]

Pharmacophore models for nicotinic agonists have been proposed for four decades. Central to these models is the presence of a cationic nitrogen and a hydrogen bond acceptor. It is now well established that the cationic center makes an important cation-π interaction to a conserved tryptophan, but the donor to the proposed hydrogen bond acceptor has been more challenging to identify. A structure of nicotine bound to the acetylcholine binding protein (AChBP) predicted that the binding partner of the pharmacophore's second component was a water molecule, which also hydrogen bonds to the backbone of the complementary subunit of the receptors. Here we use unnatural amino acid mutagenesis coupled with agonist analogs to examine whether such a hydrogen bond is functionally significant in the α4β2 neuronal nAChR, the receptor most associated with nicotine addiction. We find evidence for the hydrogen bond with the agonists' nicotine, acetylcholine, carbamylcholine and epibatidine. These data represent a completed nicotinic pharmacophore and offer insight into the design of new therapeutic agents that selectively target these receptors.

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316. Altered activity-rest patterns in mice with a human autosomal-dominant nocturnal frontal lobe epilepsy mutation in the β2 nicotinic receptor

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High-affinity nicotinic receptors containing beta2 subunits (β2*) are widely expressed in the brain, modulating many neuronal processes and contributing to neuropathologies such as Alzheimer's disease, Parkinson's disease and epilepsy. Mutations in both the α4 and β2 subunits are associated with a rare partial epilepsy, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Here we introduced one such human missense mutation into the mouse genome to generate a knock-in strain carrying a valine-to-leucine mutation β2V87L. $\beta2^{V287L}$ mice were viable and born at an expected Mendelian ratio. Surprisingly, mice did not display an overt seizure phenotype; however, homozygous mice did display significant alterations in their activity-rest patterns. This was manifest as an increase in activity during the light cycle suggestive of disturbances in the normal sleep patterns of mice; a parallel phenotype to that found in human ADNFLE patients. Consistent with the role of nicotinic receptors in reward pathways, we found that $\beta2^{V287L}$ mice did not develop a normal proclivity to voluntary wheel running, a model for natural reward. Anxiety-related behaviors were also affected by the V287L mutation. Mutant mice spent more time in the open arms on the elevated plus maze (EPM) suggesting that they had reduced levels of anxiety. Together, these findings emphasize several important roles of β2* nicotinic receptors in complex biological processes including the activity-rest cycle, natural reward, and anxiety.

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317. Probing the binding interaction of cytisine and varenicline to the low affinity α4β2 nAChR

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The α4β2 nicotinic acetylcholine receptor (nAChR) is a pentameric neuronal ligand-gated ion channel (LGIC) that exists in two stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$, termed the high and low affinity receptors, respectively. Our group has previously shown that nicotine binding to the high affinity α4β2 nAChR is mediated, in part, through a cation-π interaction at a conserved tryptophan residue (αW154) in loop B of the extracellular domain, as well as a hydrogen bond to the backbone carbonyl at αW154. Cytisine is a partial agonist at the α4β2 nAChR and selectively activates $(\alpha4)_3(\beta2)_2$ over $(\alpha4)_2(\beta2)_3$. This compound has been used as a smoking cessation drug and served as inspiration for the development of varenicline (Chantix®). We plan to study binding interactions of both cytisine and varenicline with the $(\alpha4)_3(\beta2)_2$ nAChR. Through the use of unnatural amino acid mutagenesis and nonsense-suppression, a series of fluorinated unnatural amino acids will be systematically introduced at αW154, as well as other aromatic residues in the ligand binding domain of the $(\alpha4)_3(\beta2)_2$ receptor, to probe the existence of a cation-π interaction. The hydrogen bond interaction to the backbone carbonyl at αW154 will be examined by substituting the i + 1 residue, αT155 for threonine α-hydroxy acid (Tah), thus weakening the carbonyl of interest as a hydrogen bond acceptor. These experiments will further our understanding of agonist binding to $(\alpha4)_3(\beta2)_2$ nAChR.

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318. Alpha5 subunits in alpha4beta2 nicotinic receptors: assembly and function

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Nicotinic acetylcholine receptors (nAChRs) are ligand gated ion channels abundantly expressed in the peripheral and central nervous system. Nicotine is the major addictive component of tobacco and chronic tobacco use (smoking) has been linked to many types of cancer as well as heart disease. Other related phenomena include an inverse correlation between smoking and Parkinson's disease and the observation that patients with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) who smoke have fewer seizures. Recently, several genome-wide association and candidate gene studies have identified polymorphisms in the gene for the α5 nAChR subunit that are linked to an increased risk for nicotine addiction, alcohol addiction, and lung cancer. This report describes preliminary studies on heterologously expressed receptors containing the α5 subunit (α5* nAChRs). The nAChRs belong to the Cys-loop family of ionotropic receptors, which share a pentameric architecture of subunits arranged around a central ion permeable pore. Many distinct subunit combinations can form receptors. Using high-resolution fluorescence microscopy techniques including Förster resonance energy transfer (FRET) and total internal reflection fluorescence (TIRF) we are able to visualize the nAChR's life cycle from assembly to degradation, within living cells. I will now characterize α4β2α5 receptor stoichiometry and trafficking in N2a cells and in primary mouse midbrain neurons using high-resolution fluorescence microscopy and electrophysiology. I will characterize the effects of including the mutant α5D398N on α4α2 receptor stoichiometry and trafficking using high-resolution fluorescence microscopy and electrophysiology. I will determine the affect of 0.4, 4, and 40 h exposure to nicotinic drugs on the stoichiometry and trafficking of α4β2α5 or α4β2α5-D398N receptors in N2a cells and primary mouse midbrain neurons. The aim of this research is to uncover the molecular and subcellular processes that may underlie the mechanism through which α5* receptors may contribute to disease and addiction states.

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319. Cholinergic modulation of locomotion and striatal dopamine release is mediated by α6α4* nicotinic acetylcholine receptors

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Dopamine (DA) release in striatum is governed by firing rates of midbrain DA neurons, striatal cholinergic tone, and nicotinic ACh receptors (nAChRs) on DA presynaptic terminals. DA neurons selectively express α6* nAChRs, which show high ACh and nicotine sensitivity. To help identify nAChR subtypes that control DA transmission, we studied transgenic mice expressing hypersensitive $\alpha6^{L9'S}$* receptors. $\alpha6^{L9'S}$ mice are hyperactive, travel greater distance, exhibit increased ambulatory behaviors such as walking, turning, and rearing, and show decreased pausing, hanging, drinking, and grooming. These effects were mediated by α6α4* pentamers, as $\alpha6^{L9'S}$ mice lacking α4 subunits displayed essentially normal behavior. In $\alpha6^{L9'S}$ mice, receptor numbers are normal; but loss of α4 subunits leads to fewer and less sensitive α6* receptors. Gain-of-function nicotine-stimulated DA release from striatal synaptosomes requires α4 subunits, implicating α6α4β2* nAChRs in $\alpha6^{L9'S}$ mouse behaviors. In brain slices, we applied electrochemical measurements to study control of DA release by $\alpha6^{L9'S}$ nAChRs. Burst stimulation of DA fibers elicited increased DA release relative to single action potentials selectively in $\alpha6^{L9'S}$, but not WT or α4KO/$\alpha6^{L9'S}$, mice. Thus, increased nAChR activity, like decreased activity, leads to enhanced extracellular DA release during phasic firing. Bursts may directly enhance DA release from $\alpha6^{L9'S}$ presynaptic terminals, as there was no difference in striatal DA receptor numbers or DA transporter levels or function *in vitro*. These results implicate α6α4β2* nAChRs in cholinergic control of DA transmission, and strongly suggest that these receptors are candidate drug targets for disorders involving the DA system.

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320. Optimization of glutamate-gated chloride channels as a tool for neuronal silencing via protein engineering

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The development of novel pharmacological-genetic techniques to manipulate excitability is a major goal in modern neuroscience. We previously engineered a fluorescently tagged glutamate-gated chloride (GluCl) channel that can silence electrical activity in targeted CNS neurons *in vivo* when activated by ivermectin (IVM). However, the IVM concentration required to achieve a consistent silencing phenotype is high enough to raise

concerns about its potential side effects. To increase agonist sensitivity, a conserved leucine (9' position) in the second transmembrane region of the GluClα subunit was mutated to each of seven other residues. Glutamate concentration-response relations of the fluorescently tagged, heteromeric wild-type (WT) and mutant channels were obtained in HEK293 cells by whole-cell patch clamp with millisecond microperfusion. The WT EC_{50} was 180 ± 30 μM (mean ± SEM, n = 12 cells). Comparing EC_{50}, all mutations (L9'S, A, F, I, T, V) except L9'G significantly increased glutamate sensitivity by a factor of 5- to 80-fold. Their median peak responses were 16% (L9'A) to 43% (L9'G) of WT. Several L9' mutations increased background conductance, suggesting a higher probability of unliganded openings. The L9'F mutation substantially increased glutamate sensitivity (8-fold) without a noticeable increase in background conductance and displayed 31% of WT maximum response. To compensate for the reduced maximum response, we attempted to improve GluCl surface expression by removing endoplasmic reticulum (ER) retention motifs in the intracellular loop of the β subunit. Preliminary fluorescence imaging data suggest that mutating the RSR and RRR motifs to AAA dramatically increases GluCl expression at the plasma membrane. Thus, we hope that combining the L9'F and ER retention mutations will produce a functionally optimized GluCl/IVM neuronal silencing tool.

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321. Structural differences determine the relative selectivity of nicotinic compounds for native α4β2*-, α6β2*-, α3β4*- and α7-nicotine acetylcholine receptors

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Mammalian brain expresses multiple nicotinic acetylcholine receptor (nAChR) subtypes that differ in subunit composition, sites of expression and pharmacological and functional properties. Among known subtypes of receptors, the α4β2* and α6β2*-subtypes have the highest affinity for nicotine (where *indicates possibility of other subunits). The α4β2*-nAChRs are widely distributed, while the α6β2*-subtypes are restricted to a few regions. Both subtypes modulate release of dopamine from the dopaminergic neurons of the meso-accumbens pathway thought to be essential for reward and addiction. The α4β2*-subtype also modulates GABA release in these areas.

Identification of selective compounds would facilitate study of nAChR subtypes. An improved understanding of the role of nAChR subtypes may help in developing more effective smoking cessation aids with fewer side effects than current therapeutics. We have screened a series of nicotinic compounds that vary in the distance between the pyridine and the cationic center, in steric bulk, and in flexibility of the molecule. These compounds were screened using membrane binding and synaptosomal function assays to determine affinity, potency and efficacy at four subtypes of nAChRs found in brain, α4β2*, α6β2*, α7 and α3β4*. In addition, physiological assays in gain-of-function mutant mice were used to assess *in vivo* activity at the α4β2* and α6β2*-subtypes. This approach has identified several compounds with agonist or partial agonist activity that display improved selectivity for the α4β2*-nAChR.

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322. The use of fluorescence microscopy to determine the interaction of P2X channels with nicotinic receptors

Pallavi Gunalan, Henry A. Lester

Neuronal nicotinic acetylcholine receptors (nAChRs) are members of the Cys-loop family cationic channels and are triggered by acetylcholine as well as nicotine, the addictive substance found in cigarettes. Understanding the assembly and trafficking of these pentameric structures is integral to understanding the mechanism behind addiction. These receptors exhibit a cross-inhibitory action in the presence of P2X receptors, which are ATP-gated cation channels. We are studying the role that the direct physical interaction between these receptors plays in the observed cross-inhibition, and how this influences the assembly, stoichiometry, and trafficking of nAChRs. Förster resonance energy transfer (FRET) reveals detailed information on intracellular interactions between P2X and either nascent nicotinic receptors, or even unassembled subunits. Single particle tracking using quantum dots reveals the effect this phenomenon has on simultaneous lateral diffusion at the cell surface. We are currently using these techniques to investigate the influence of P2X2 receptors on the assembly and trafficking of α4β2 nAChRs to determine whether interactions originate intracellularly, or whether they begin at the cell membrane.

323. Total internal reflection fluorescence microscopy and single molecule fluorescence reveal GAT1 vesicle dynamics and GAT1 density on vesicles

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While the study of vesicle fusion has been extensively applied to neurotransmitter- and neuropeptide-containing vesicles, there is evidence that secretory vesicles have a complement of proteins distinct from the vesicles that traffic membrane proteins. These differences in the vesicle composition suggest inherent differences in trafficking mechanisms, which can be studied by characterizing membrane protein trafficking. To this end, we applied total internal reflection fluorescence microscopy (TIRFM) to quantify the density of GAT1 molecules on vesicles, using a well-characterized N2a cell expression system. We also analyzed the dynamics of vesicles containing fluorescently tagged GAT1. We determined that 3-7 molecules of GAT1 are present per vesicle. Images of GAT1 vesicles undergoing plasma membrane fusion revealed similarities with previous reports on other vesicles. We also identified a population of GAT1 vesicles with ATP-dependent lateral displacement.

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324. Molecular interaction between nicotinic acetylcholine receptors and the modulator protein lynx1

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Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels expressed throughout the brain and at neuromuscular junctions. These receptors are homo- or hetero- pentameric with homologous subunits arranged around a central ion pore. The lynx family-of-proteins have been shown to physically associate with nAChRs and are expressed in brain areas heavily involved in nicotinic function. Lynx modulators are thought to support proper nAChR function *in vivo*.

Molecular-scale interactions between the lynx proteins and the nAChR are the target of our investigation. Specifically, we seek to identify the sites of interaction between nAChR and lynx1, the first protein discovered of the family. Lynx1 is a small protein containing 72 amino acids with a C-terminal glycophosphoinositide-linked (GPI-linked) sequence. Lynx's cysteine-rich motif is characteristic of the class of elapid snake venom neurotoxins such as α-bungarotoxin (αBtx) and cobratoxin, which are known competitive antagonists to specific nAChR subtypes. The GPI-linked motif would topologically allow the lynx proteins to bind in a similar fashion to αBtx at the intersubunit interface on nAChR. However, the antagonistic effect, as seen with the toxins, has not been demonstrated with lynx binding. This raises the possibility that lynx binds to the non-agonist interfaces of the receptor that are allosterically important to gating. Our preliminary electrophysiology results in *Xenopus* oocytes reveals different effects of lynx1 on the $(\alpha4)_3(\beta2)_2$ and $(\alpha4)_2(\beta2)_3$ stoicheometry of the α4β2 nAChR, although their agonist binding interfaces are identical. With photobleaching experiments on total internal reflection (TIRF) microscopy, we aim to determine whether or not lynx1 binds to nAChR at the agonist binding interfaces (as αBtx does) and to identify the number of lynx1 binding sites per receptor. Once the broad regions of the binding site are located, we hope to focus further onto specific binding residues using site-directed mutagenesis.

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325. A cation-π interaction at a phenylalanine residue in the glycine receptor-binding site is conserved for different agonists

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Cation-π interactions have been demonstrated to play a major role in agonist binding in Cys-loop receptors. However, neither the aromatic amino acid contributing to this interaction, nor its location is conserved among different Cys-loop receptors. Similarly, it is not clear how many different agonists of a given receptor form a cation-π interaction, or, if they do, whether it is with the same aromatic amino acid as the major physiological agonist. We previously demonstrated that Phe159 in the glycine receptor (GlyR) α1 subunit forms a strong cation-π interaction with the principal agonist, glycine. In this study we investigated whether the lower efficacy agonists of the human GlyR, β-alanine and taurine, also form cation-π interactions with Phe159. By incorporating a series of unnatural amino acids we found cation-π interactions between Phe159 and the amino groups of β-alanine and taurine. The strengths of these interactions were significantly weaker than for glycine. Modeling studies suggest that β-alanine and taurine are orientated subtly differently in the binding pocket, with their amino groups further from Phe159 than that of glycine. These data therefore, show that similar agonists can have similar but not identical orientations and interactions in the binding pocket, and provide a possible explanation for the lower potencies of β-alanine and taurine.

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326. Investigating the upregulation of the α6 nAChR subunit in response to chronic nicotine using a modified bacterial artificial chromosome (BAC)

Elisha Mackey, Ryan M. Drenan, Henry A. Lester

Receptors containing α6 nicotinic acetylcholine receptor (nAChR) subunits have the highest sensitivity to nicotine yet measured; therefore, α6 could play an important role in understanding nicotine addiction. To investigate upregulation of the α6 nAChR subunit in response to chronic nicotine, we are visualizing receptors containing α6 in a mouse line expressing fluorescently labeled α6 and observe changes in expression in response to nicotine. A GFP sequence was inserted into the M3-M4 loop on exon 5 of Chrna6 (α6 nAChR) located on a 156kb BAC using recombination in a two-step selection and counter selection technique. The BAC includes significant 5' and 3' flanking regions for faithful expression of α6. The modified BAC was then randomly introduced into the mouse genome via pronuclear injection of fertilized mouse eggs. We have now identified 15 founder mouse lines.

We confirm the expectation that α6* nAChRs are expressed in nearly all dopaminergic cells of the ventral midbrain. We also expected to find α6* nAChRs in the retinal ganglion cells, optic nerve, and the synaptic terminals of the optic nerve in the superior colliculus. This expectation is satisfied; however, the data also reveal that α6* nAChRs are expressed in nearly all retinal ganglion cells. We add the unexpected information that α6* nAChRs are also expressed in postsynaptic cells of the superior colliculus, and that these expressing neurons are probably GABAergic.

Mice that express the transgene at single-copy numbers will be homozygosed and then crossed to an α6 knock-out line; thus we expect to have strains with wild-type numbers of fluorescent α6* nAChRs. The mice will then be exposed to chronic nicotine or chronic saline for ten days using osmotic mini-pumps, thereafter, the brains will be sectioned and examined for changes in levels of α6 receptor subunit expression. These results will provide insight into the relationship between specific changes in number and distribution of nicotinic receptors and nicotine thus, contributing to our understanding of the role of α6 receptors in nicotine addiction.

327. Role of murine Lynx1 in nicotine-mediated neuroprotection of mouse cortical neurons

Mahati Mokkarala, Julie Miwa, Rahul Srinivasan, Henry A. Lester

Previous studies have indicated that although removal of novel murine protein lynx1 promotes greater synaptic plasticity for mouse cortical neurons, complete removal of lynx1 also reduces nicotine's neuroprotective effects against excitatory cell death. We therefore, want to determine if lynx1 heterozygous mice can maintain nicotine-mediated neuroprotection by using a live/dead cell assay system. The cell assay differentiates between live and dead cell by using fluorescent compounds calcein-AM and ethidium homodimer for live and dead cells, respectively. Preliminary analysis of wild-type cortical neuron samples with nicotine and glutamate indicates that the assay detects effects of nicotine-mediated neuroprotection against excitotoxicity. The assay can therefore, be useful for quantitative analysis on how heterozygous lynx1 samples interact with nicotine neuroprotection. We hope that the assay system can explain the mechanisms of nicotine-mediated neuroprotection against glutamate-induced excitatory cell death in mouse cortical neurons.

328. A conserved proline residue at the center of transmembrane helix 12 of the γ-aminobutyric acid transporter (GAT1) is required for the correct function of C-terminal membrane targeting motifs

Fraser J. Moss, Elisha Mackey, Henry A. Lester

Oligomerization of mammalian SLC6 transporter protomers is necessary for exit from the endoplasmic reticulum. LeuT, a bacterial SLC6 homologue, crystallized as a dimer; TM12 forming a major part of the transporter dimer interface. P549 in TM12 of the mouse γ-amino butyric acid (GABA) transporter, mGAT1, is conserved in all members of the mammalian SLC6 transporter family, but not in LeuT. To determine if P549 in GAT1 was important for the assembly, trafficking and function of the mammalian SLC6 transporters we mutated the P549 residue in GAT1 to a glycine, valine or alanine. mGAT1P549V exhibited a V_{max} only 26% and a K_m more than double that for wild-type GAT1. [^{3}H]GABA uptake was barely above background for both the P549G and P549A mutations and fluorescence microscopy showed that they were expressed almost entirely in intracellular membranes whereas mGAT1P549V localized both intracellular membranes and as an annulus of fluorescence in the cell periphery. Normalized Förster resonance energy transfer (NFRET) signal versions of each P549 mutant co-expressed in N2a cells were 1.3-, 1.7 -and 2.4-fold increased compared to the wild-type functioning mGAT1XFP8 oligomers and followed the trend for increasing helix propensity for the residue at position 549; P < G < V < A. P549G and P549A whole cell and perinuclear NFRET amplitude distributions were fit by two Gaussian components characteristic for GAT1 constructs that oligomerize but which display impaired trafficking to

the plasma membrane. The NFRET amplitude distributions from all regions of interest from cells expressing the mGAT1P549V mutant were fit with three Gaussian components, indicating the mGAT1P549V oligomers escaped the ER and were present in organelles that allow the transporters to make PDZ-mediated interactions with cytoplasmic proteins. Efficient concentrative export of mGAT1 from the ER requires COPII recognition motifs in its C-termini to be present in oligomeric form. All three P549 mutants exerted a dominant-negative influence on wild-type mGAT1 function, indicating a change in TM12 helix conformation or reducing its flexibility does not impair oligomerization but impairs access to COPII recognition motifs in an mGAT1 dimer. Furthermore, we coexpressed YFP-Sec24D with wild-type functioning mGAT1CFP8 or P549G, P549V or P549A mGAT1CFP8 mutants and examined the NFRET distributions within ER exit sites, where the YFP-Sec24D concentrated as bright yellow puncta. Within these puncta, the NFRET distribution for mGAT1CFP8 and YFP-Sec24D was fit two Gaussian components; the first negative mean amplitude component represented non-interacting species with non-perfect bleed-through compensation, and the second positive mean NFRET amplitude component represented the interaction of mGAT1CFP8 with YFP-Sec24D. Both components of the NFRET signal from P549G, P549V or P549A mGAT1CFP8 mutants had negative mean amplitude, indicating that interaction of the COPII coat protein is inhibited. However, Sec24D/mGAT1 interaction was not abolished in cells expressing P549G or P549A mutants because pixels with positive mean NFRET amplitudes did exist. Furthermore, more pixels with positive NFRET existed the distribution for the mGAT1P549V-CFP8/YFP-Sec24D co-expression. This made the mean amplitude of the single Gaussian component in the distribution less negative than for P549G or P549A mutants with YFP-Sec24D. The interaction of YFP-Sec24D with mGAT1P549V-CFP8 was therefore, inefficient, but less so than for P549G or P549A mutants. In summary, the conserved P549 residue of mGAT1, introduces a conformation into TM12 of mammalian SLC6 transporters not predicted by the LeuT crystal structure that is essential to allow efficient interaction of SLC6 dimers with the COPII ER export machinery and their efficient export from the ER out to the plasma membrane.

329. Coassembly of nicotinic acetylcholine receptor (nAChR) α7 and β2 subunits, and possible roles of RIC-3 and β2 subunits in α7*-nAChR function and trafficking

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Nicotinic acetylcholine receptor (nAChR) α7 subunits, found in central and peripheral nervous systems, form homopentameric receptors in heterologous expression systems. However, heteromeric α7*-nAChRs have been less well studied in mammalian cell expression systems. Fluorescently-tagged mouse α7 and β2 subunits, each with a unique fluorescent protein (FP) integrated into the subunit's second, large intracellular loop (C2), were stably coexpressed in native nAChR-null, human SH-EP1 cells. Fluorescence confocal microscopy showed that both FP-subunits were predominantly localized intracellularly. Confocal microscopy showed intracellular colocalization, and total internal reflection fluorescence (TIRF) microscopy revealed colocalization in plasma membrane-rich filopodia-like processes. Moreover, we show for the first time that Förster resonance energy transfer (FRET) occurred between the fluorescently-tagged subunits, including in plasma membrane regions, demonstrating coassembly. Exposure to choline produced inward currents in cells transfected with tagged α7 and β2 subunits, but at ~ half the level of response in cells transfected with α7 subunits alone. Coexpression of human RIC-3 (hRIC-3), a chaperone protein known to enhance functional expression of homopentameric α7-nAChRs, resulted in a doubling of current amplitudes in cells expressing fluorescently-tagged α7 subunits either alone or in combination with tagged β2 subunits. Remarkably, hRIC-3 coexpression markedly reduced expression of both FP-subunits in filopodia-like processes, suggesting that hRIC3 alters subunit trafficking with subcellular specificity. Taken together, these results are consistent with coassembly of nAChR α7 and β2 subunits, perhaps to form α7β2-nAChRs, confirm heterologous expression of functional nAChR containing fluorescently-tagged α7 subunits, and suggest roles for nAChR β2 subunits and hRIC-3 in α7*-nAChR assembly, trafficking, and function.

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330. The role of lynx1 in cellular membrane trafficking of nicotinic acetylcholine receptors

Rell L. Parker, Julie M. Miwa, Henry A. Lester

Lynx1 is a protein modulator of nicotinic acetylcholine receptors (nAChRs); the goals of the projects detailed here are to assess the interactions of lynx proteins and nAChRs, and determine the consequence that this interaction has on the trafficking behavior of nAChRs. Lynx1 is a member of the glycosyl phosphatidylinositol (GPI)-anchored protein class, these proteins are linked to cellular membranes by lipophilic anchor. The GPI anchor is known to mediate targeting to specific regions of lipids that are high in cholesterol and sphingolipids, termed lipid rafts. The first goal of the project is to determine whether lynx1 is important for mediating localization of nAChRs in cellular membrane trafficking.

Lipid rafts are believed to bring various proteins into close proximity to affect downstream signaling pathways. We hypothesize that lynx1 binds nAChRs and cause them to be localized into lipid rafts. Our hypothesis will be tested using a combination of confocal and total internal reflection microscopy (TIRFM). Using cholera toxin B (CT-B), which has affinity to sphingolipids, we plan to determine if fluorescently tagged nAChRs are colocalized with lipid rafts. Additionally, we plan to use fluorescence recovery after photobleaching (FRAP) to monitor the mobility of nAChRs, as proteins in lipid rafts are known to have a lower mobility. The studies described will be completed in N2A cells, but we also have interest in studying the trafficking of nAChRs in lynx1 knockout neurons as compared to wild-type neurons.

A second goal of the project is to determine whether some of the differences in receptor localization can be attributable to lynx:receptor interactions within the cell interior. Since interactions between stable complexes of GPI-proteins with membrane proteins can occur as early as the Golgi, lynx proteins have the potential to bind at an early-stage in the sorting pathway. For these studies, we carried out a combination of high-resolution microscopic assays in real-time in living cells, again relying on fluorescently tagged receptor moieties to track the receptors. Two types of FRET measurement were conducted on cells transfected with $\alpha4\beta2$ nAChRs or $\alpha4\beta2$ plus lynx1. Donor recovery after photobleaching (DRAP) measurements and pixel-by-pixel NFRET measurements indicates a significant shift in the FRET efficiency values due to the presence of lynx1. Furthermore, imaging in TIRF mode indicates both static and dynamic differences in receptor patterns in the ER. Lastly, in primary neuronal cultures, transfection of fluorescently tagged $\alpha4$ and $\beta2$ constructs into cortical neurons from wild-type and lynx1KO, indicate an alteration in the distribution of nAChRs to pools of high density receptors at dendritic branch points, due to the absence of lynx1. There was no effect on the absolute number of receptors. Taken together, our data indicate that the interaction of lynx1: nAChRs can restrict receptor trafficking, and that one of the mechanisms by which lynx achieve modulatory capabilities on the nicotinic receptor system is through nAChR localization and assembly.

331. $\alpha4$* nAChR modulation of glutamate release from the medial perforant path

Rachel E. Penton, Sheri McKinney, Henry A. Lester

Nicotine interacts with nicotinic acetylcholine receptors (nAChRs) in several brain areas outside of the classical reward system and is involved in the release of other neurotransmitters in addition to stimulating dopamine release. This may be important in the medial perforant path (MPP) projection from medial entorhinal cortex (MEC) to dentate gyrus (DG), a pathway that may be involved in the cognitive effects associated with chronic nicotine exposure. Previously our lab has shown that $\alpha4$* nAChRs are upregulated on MPP axons following chronic nicotine treatment and activation of these receptors facilitates long-term potentiation induction, presumably by increasing glutamate release (Nashmi *et al*., 2007). Here we have begun to investigate how upregulation of $\alpha4$* nAChRs located on MPP axons modulates glutamate release.

In acutely prepared hippocampal slices from mice treated with either nicotine or saline for one day, whole-cell patch clamp recordings of spontaneous excitatory postsynaptic currents (sEPSCs) from DG granule cells, which are postsynaptic to the MPP axons, were made in the presence of bicuculline (10 μM). Acute application of nicotine (1 μM) increased the frequency of sEPSCs to ~130% of baseline in cells from nicotine-treated animals (nic-cells, n = 8), but did not alter the frequency of sEPSCs in cells from saline-treated animals (sal-cells, n = 7) indicating upregulation of the nAChRs. This increase in sEPSC frequency is not likely the result of acute nicotine desensitizing upregulated $\alpha4$* nAChRs as the baseline sEPSC frequencies did not differ and DHβE (300 nM) did not mimic the effect of nicotine, having no effect on the frequency of sEPSCs in either treatment group (nic-cells, n = 7; sal-cells, n = 8). Recordings of sEPSCs do not allow disambiguation between responses from lateral perforant path (LPP) and MPP thus, we are currently examining the effects of acute nicotine on evoked EPSCs. These studies will give insight to the manner in which upregulated $\alpha4$* nAChRs modulate glutamate release from MPP axons, a possible mechanism underlying the cognitive effects of nicotine.

332. Using unnatural amino acids to study the ligand-binding mechanism of the α4β4 and α7 nAChRs

Nyssa Puskar, Xinan Xiu, Dennis D. Dougherty*, Henry A. Lester*

Nicotinic acetylcholine receptors (nAChR) are pentameric neurotransmitter-gated ion channels responsible for rapid excitatory neurotransmission in the central and peripheral nervous systems, resulting in skeletal muscle tone and various cognitive effects in the brain. These complex proteins are activated by the endogenous neurotransmitter acetylcholine (ACh), as well as nicotine and structurally-related agonists. Activation and modulation of nAChRs has been implicated in the pathology of multiple neurological disorders, and as such, these proteins are potential therapeutic targets. Here we use unnatural amino acid mutagenesis to examine the ligand binding mechanism of two homologous neuronal nAChRs, the α4β4 and α7 receptors. While sequence identity among the residues that form the agonist binding site is completely conserved for both receptors, we find that that the α4β4 and α7 nAChRs employ different agonist-receptor binding interactions. The α4β4 receptor utilizes a strong cation-π interaction to a conserved tryptophan (TrpB) of the receptor for both ACh and nicotine. We also report that nicotine participates in a strong hydrogen bond with a backbone carbonyl contributed by TrpB in α4β4. Interestingly, we find that the α7 receptor also employs a cation-π interaction for ligand-recognition, but the site has moved to a different aromatic amino acid of the agonist-binding site depending on the agonist. ACh participates in a cation-π interaction with TyrA, while epibatidine participates in a cation-π interaction with TyrC2.

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333. Optimization of protocols to differentiate human embryonic stem cells into dopaminergic neurons

Doreen Rhee, Rahul Srinivasan, Steven Goldman, Henry A. Lester*

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by the selective cell death of dopaminergic neurons of the substantia nigra. Epidemiological studies have documented an inverse correlation between PD and smoking. Nicotine, the active ingredient in cigarettes, is thought to contribute to this effect.

Attempts to study dopaminergic neurons *in vitro* have been hampered by poor yield and survival of primary neurons in culture. The aim of the current study is to address this bottleneck by optimizing methods to differentiate and culture the particular subtype of DA neurons affected in PD. Neuronal differentiation of human embryonic stem cells (hESC) was achieved using previously described techniques.

Pluripotent hESC colonies were mechanically dissociated and cultured in suspension to form embryoid bodies (EBs). Culture as EBs induces the loss of pluripotency. To encourage neuralization and a restricted differentiation into neural stem cells, EBs were plated and grown using a substrate for attachment in defined media. Cells were committed to specific neural lineage subtypes after several days in culture by adding fresh media including specific growth factors and signaling molecules. Differentiated neurons were then allowed to mature in culture, the entire process taking approximately two months. Neuronal differentiation was confirmed by immunostaining for the neuron specific antibody Tau-1.

Experiments are currently underway to optimize dopaminergic neuron differentiation protocols. Future studies will attempt to transiently express fluorescently labeled nicotinic acetylcholine receptors in these neurons. High resolution fluorescent microscopy will be used to study the effects of nicotine on receptor assembly and trafficking in dopaminergic neurons as it relates to PD.

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334. Single event imaging of nicotinic receptor dynamics

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Advances in fluorescence imaging have improved our understanding of the intracellular dynamics of nicotinic receptors, but several facets of the complex mechanisms of nicotine induced nAChR upregulation remain undefined. Upregulation of α4β2, a specific receptor subtype, has been established as an integral component of both nicotine dependence and the neuroprotective effect of nicotine against Parkinson's disease. Still, two major challenges complicate our understanding of receptor dynamics of these important biological problems. First, existing technology limits efforts to distinguish between different stoichiometries at the individual receptor level while simultaneously measuring functionality such as opening, closing, and desensitization of channels. Additionally, single-channel patch clamping, which is ideal for measuring single receptor gating events, is not capable of simultaneously measuring multiple receptors and also gives limited information about channel and receptor kinetics in neuronal processes (axons and dendrites) due to spatial limitations of the technology. Understanding these events in terms of the dynamics of the intra-cellular redistribution that results from nicotine exposure requires the study of the kinetics that drive upregulation. Single event fluorescence analysis provides insight into the dynamics of biological systems that are often obscured with ensemble averaging of bulk fluorescence. We are developing new imaging methods designed to correlate two important parameters: high-resolution single event techniques to identify receptor subtypes and stoichiometries with simultaneously measured receptor function through single channel Ca^{2+} flux. Single photon counting to time stamp each individual emitted photon allows high temporal

resolution capable of monitoring gating of individual receptors within neuronal processes. This provides a novel method for the direct measurement of receptor kinetics even at the synapse. In order to study the effect of nicotine on the trafficking of receptors to the membrane, single event imaging is used to track vesicle arrivals in neurites directly measuring insertion kinetics. These measurements in the presence and absence of agonists reveal the dynamics of nicotine-induced upregulation by monitoring variations in trafficking rates. Ecliptic pHluorin, a pH sensitive fluorescent protein, allows us to isolate individual membrane insertion events to determine the kinetics driving population of receptors in the cell membrane both in the presence and absence of nicotine. Determining the kinetics for individual events such as Ca^{2+} flux through individual receptors, single molecule FRET of membrane inserted receptors, and vesicle tracking requires the use of a variety of existing and emerging single event imaging techniques.

335. Biophysics of ligand-gated ion channel selectivity: insights through engineering a cation-selective ivermectin sensitive channel for neuronal activation

Akram S. Sadek[*], Henry A. Lester

Opto-genetic and pharmacological-genetic techniques are an emerging technology that allows the investigation of neural circuits *in vivo* through the control of membrane excitability in targeted neurons. It was previously shown that a nematode glutamate-gated chloride channel (GluCl) could be engineered to silence mammalian neurons *in vivo* through the action of the macrocyclic lactone ivermectin (IVM). Although cation-selective light activated channels have been developed to stimulate neurons, a pharmacologically activated cation selective channel has yet to be developed for use in neuroscience. We present our results on re-engineering the GluCl channel to confer cationic selectivity through mutations in the M1-M2 domain, with the goal of developing an IVM sensitive excitatory channel for the study of neural circuits in behaving animals. We investigate the biophysics of Na^+, K^+ and Cl^- selectivity in ligand-gated channels using single-channel recording, microperfusion and mutagenesis with the aim of developing general principles for engineering selectivity in this class of channels.

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336. Nicotine upregulates plasma membrane α4β2 receptors by chaperoning a stoichiometry that would otherwise languish in the endoplasmic reticulum

Rahul Srinivasan, Rigo Pantoja, Fraser J. Moss, Elisha Mackey, Cagdas D. Son, Julie Miwa, Henry A. Lester

Upregulation of α4β2* nicotinic acetylcholine receptors (nAChRs) by chronic nicotine, a cell-delimited process, may be necessary and sufficient for the initial events of nicotine dependence. Upregulation may also underlie an apparent neuroprotective effect of chronic nicotine: the inverse relationship between a person's history of tobacco use and his/her susceptibility to Parkinson's disease. To study mechanisms of upregulation, we employed transfected fluorescent protein (FP)-tagged α4 nAChR subunits. Total internal reflection fluorescence microscopy shows that nicotine (0.1 μM, 48 h) upregulates α4β2 nAChRs at the plasma membrane (PM), despite increasing the fraction of α4β2 nAChRs that remain in near-PM endoplasmic reticulum (ER). Pixel-resolved normalized Förster resonance energy transfer microscopy between α4-FP subunits shows that nicotine stabilizes the $(\alpha4)_2(\beta2)_3$ stoichiometry before the nAChRs reach the Golgi apparatus. To aid in mechanistic analysis, we generated a $\beta2_{\text{enhanced-ER-export}}$ subunit that mimics two ER-trafficking motifs of the β4 subunit sequence. The $\alpha4\beta2_{\text{enhanced-ER-export}}$ nAChR resembles nicotine-exposed nAChRs with regard to stoichiometry, intracellular mobility, and PM localization; nicotine produces only small additional PM upregulation. The data are simulated by a model incorporating two mechanisms. (1) Nicotine acts as a stabilizing pharmacological chaperone for nascent α4β2 nAChRs in the ER, eventually increasing PM receptors despite a bottleneck(s) in ER export. (2) Removal of the bottleneck, (e.g., by expression of the $\beta2_{\text{enhanced-ER-export}}$ subunit), is sufficient to increase PM nAChR numbers even without nicotine. The nicotine-nAChR interaction also induces additional ER exit sites (visualized by FP-tagged Sec24D), suggesting that pharmacological chaperoning of nAChRs by nicotine can generally enhance ER exit processes.

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337. Chronic nicotine modulates the interaction between ventral temental area and amygdala

Jackie Tan, Cheng Xiao, Ying Wang, Henry A. Lester

Nicotine is a bioreactive substance in tobacco that incurs addictive use through modified neuronal activity and interactions in the brain. Chronic exposure maintains this neuroadaptation and withdrawal disrupts the homeostasis. The ventral tegmental area (VTA) and the amygdala are implicated in nicotine reward and withdrawal respectively. In order to test whether chronic nicotine could change the communication between VTA dopaminergic (DA) neurons and the amygdala, miniosmotic pumps were subcutaneously inserted into C57BL/6 mice to chronically apply nicotine or saline for 10-14 days, followed by live brain slice patch clamp recording on VTA dopaminergic neurons. Mice treated with either nicotine (n = 2) or saline (n = 2) for 14 days and those withdrawn from either nicotine (n = 2) or saline (n = 2) for 5 days were euthanized and brain tissue was removed for immunohistochemisty. It is found that 1) in VTA, chronic nicotine decreased the firing rate of VTA DA neurons as compared to chronic saline treated VTA DA neurons ($p = 0.02$); 2) nicotine perfusion increased the firing rate of VTA DA in chronic saline treated mice, but relatively less in chronic nicotine treated mice and the difference is statistically significant ($p = 0.03$). Ongoing immunohistochemistry experiments aim to reveal chronic nicotine effects on dopamine and corticotrophin releasing factor levels in both amygdala and in VTA.

338. Probing the transmembrane proline residues of the D2 dopamine receptor with unnatural amino acids

Ethan B. Van Arnam[*], Henry A. Lester, Dennis A. Dougherty[*]

Proline residues are found within the transmembrane helices of all GPCRs, creating kinks that may play a critical role as hinges or pivot points during receptor activation. We have used unnatural amino acid mutagenesis of these proline residues in the D2 dopamine receptor to determine which specific property of proline is responsible for its function at these sites. Backbone amide-to-ester mutations using unnatural α-hydroxy acids at each transmembrane proline site establish the importance of these prolines' lack of hydrogen bond donor ability. Mutagenesis of these same sites with unnatural proline analogs suggests that transmembrane proline cis-trans isomerization is not a key event in receptor activation.

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339. *In vivo* distinction between plasma membrane and near-membrane organelles using Variable Angle Total Internal Reflection Fluorescence Microscopy (VA-TIRFM)

Lawrence Wade, Chris Richards, Rahul Srinivasan, Daniel Axelrod[*], Henry A. Lester

We have imaged cellular membranes in near isolation from cell organelles using a Variable Angle-Total Internal Reflection Microscope (VA-TIRFM). Further we have captured 'z-sectioned' pictures of cells only 250 nm thick. This provides 3D information similar to that gained by confocal microscopes but with at least an order-of-magnitude greater z resolution. Key to achieving this resolution was integration of a controllable excitation laser micropositioner into a standard through-the-lens TIRF illuminator and development of a custom culture dish for re-use of expensive high index of refraction cover slips. Images are acquired at several penetration depths by varying the excitation laser illumination angles. At the shallowest penetration depth (~46 nm) just the membrane and a few internal puncta are imaged. As the penetration depth is increased up to 250 nm organelles near the membrane, such as the ER, are imaged as well. The sequence of images from shallow deep is processed to yield a z-stack of images of approximately constant thickness at increasing distance from the coverslip. We employ this method to distinguish membrane-localized fluorophores (α4 GFP β2 nicotinic acetylcholine receptors and pCS2-mCherry) at the plasma membrane (PM) from those in near-PM endoplasmic reticulum (ERTracker green, α4 GFP β2 nicotinic acetylcholine receptors), on a z-axis distance scale of ~45 to ~250 nm in N2a cells. In doing so we observe occasional smooth ER structures that cannot be resolved as being distinct from the membrane.

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340. Human CHRNA7 syntenic mouse model

Ying Wang, Henry A. Lester

Emerging evidence has shown the impacts of α7 nicotinic acetylcholine receptor (α7 nAChR) on schizophrenia and suggests its potential role as a therapeutic target for cognitive dysfunction in the illness. Some polymorphisms identified in human α7 nAChR are associated with schizophrenia. However, it is challenging to determine the role of these mutations in the pathogenesis of the disease using mouse models because most polymorphisms occur in the regions that mouse gene is only partially conserved with human gene. Thus, we will make a new mouse model in which the full mouse CHRNA7 gene is syntenically replaced with human gene by recombinase-mediated cassette exchange (RMCE). The bioinformatic analysis performed on the mouse CHRNA7 locus suggests the RMCE sites to be at ~8 kb upstream of exon 1 and ~10 kb downstream of exon 11. The construction of the targeting vector for RMCE sites on mouse gene is in progress at GenOway. The bacterial artificial chromosome (BAC) RP11-494O02, which will be used to deliver human gene, contains the entire CHRNA7

gene (~125kb) as well as ~28 kb additional 5' sequence and ~10 kb additional 3' sequence. Based on the structural analysis performed on human CHRNA7, we will design our RMCE sites in regions 16.5-13.5 kb upstream of exon 1 and 9.5-11.5 kb downstream of exon 10. The BAC has initially been assessed using PCR. PCR primers are designed to amplify a 600 bp fragment that covers the M3-M4 loop of the receptor as well as a 300 bp fragment that covers the stop codon. Both PCRs result in products of expected length, indicating that the BAC does contain the gene of interest. We have ordered shotgun sequencing for the BAC to identify mutations that we can correct before using the BAC to deliver human CHRNA7 gene. The assembly of sequencing reads results in ~30 gaps which we will close by PCR approaches and primer walking steps.

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341. Association between schizophrenia and the 2bp deletion polymorphism in CHRFAM7A

Ying Wang, Cheng Xiao, Henry A. Lester

Schizophrenia is one of the major psychiatric diseases that have strong genetic elements. Multiple studies have linked the etiology of schizophrenia to chromosome region 15q13-14, a highly duplicated region that has complex rearrangements. Both α7 nicotinic acetylcholine receptor gene (CHRNA7) and its partial duplication (CHRFAM7A) map to this dynamic region. CHRFAM7A carries a 2bp deletion polymorphism (ΔTG) within exon 6, which is associated with schizophrenia. We have investigated this association by fusing fluorescent proteins, mGFP and mCherry, into the M3-M4 intracellular loop of the full-length human α7 nicotinic acetylcholine receptor (α7 nAChR)), the partial duplication of human α7 (Dα7) and the partial duplication of human α7 with 2bp deletion (DDα7). Expression of fluorescently tagged proteins is detected in transiently transfected neuroblastoma cells (N2a). The subcellular localization of Dα7 and DDα7 is similar to that of α7. Förster resonance energy transfer (FRET) reveals assembly of both Dα7 and DDα7 with α7 subunits, as well as self-assembly of Dα7 and DDα7 subunits. The interaction between the duplicated proteins (Dα7 and DDα7) and the full-length α7 subunit indicates that Dα7 and DDα7 may impact the biogenesis of α7 nAChR. To study the effect of the duplicated proteins on the functional expression of the full-length α7 nAChR, we have employed patch clamp whole-cell recordings from N2a cells expressing α7 and RIC-3 either without or with the duplicated protein. Puffs of 1 mM ACh (0.1 s) evoked inward currents with fast decay from cells transfected with α7 and RIC-3, indicating the expression of α7 receptors. Preliminary studies (n=3) show that the ACh induced currents have a different kinetics when Dα7 or DDα7 is co-expressed. The slower decay suggests that the duplicated proteins may be assembled and transported to the cell membrane together with full-length α7 subunits, and thus, contribute to the function of the channels.

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342. Synaptic connections of subthalamic neurons expressing α4β2 or α7 nAChRs

Cheng Xiao, Ying Wang, Henry A. Lester

The subthalamic nucleus (STN) constitutes the most effective target for deep brain stimulation to treat late-phase Parkinson's disease (PD). Could STN play a role in the strong inverse correlation between a person's history of tobacco use and his susceptibility to PD? Intrinsic STN glutamatergic excitatory neurons receive cholinergic innervation from pedunculopontine tegmental nucleus, and several nicotinic acetylcholine receptor (nAChR) subunit mRNAs are expressed in this region. Utilizing patch clamp whole-cell recordings from parasagittal mouse brain slices, we found that puffing 300 μM ACh (0.1 s) onto the cell body evoked inward current and accelerated firing with two decay types. The slow (Tau = 336 ± 61 ms) or fast (Tau = 54 ± 10 ms) decay occurs in 20% or 40% of STN neurons. Slow current was absent in nAChR α4 subunit knock-out (α4 KO) mice, was blocked by dihydro-β-erythroidine and activated by RJR-2403, and had the same kinetics as α4β2 nAChRs transfected in Neuron2a cells. Fast current was still present in α4 KO mice, was blocked by methyllycaconitine and activated by PNU-282987, and showed a decay phase resembling α7 nAChRs in transfected Neuron2a cells. The α4β2 nAChR expressing neurons had lower frequency of sIPSC, but higher frequency of sEPSC, than α7 nAChR expressing neurons. Dopamine depletion with reserpine significantly reduced sIPSC frequency in α4β2 (by 70%), but not in α7 expressing neurons, and changed sEPSC frequency in neither type. Interestingly, activating α4β2 nAChR in STN increased sEPSC frequency in downstream substantia nigra pars reticulata (SNr), but not in substantia nigra pars compacta (SNc), while activating α7 nAChR in STN enhanced sEPSC more strongly in SNc, than in SNr. Interestingly, chronic nicotine increased α4β2 currents evoked by both 300 and 3 μM ACh. The results suggest: 1) that α4β2 and α7 expressing neurons are controlled, with different strength, by dopaminergic, GABAergic, and glutamatergic modulation; 2) that neurons may preferentially innervate SNr neurons, while α7 expressing neurons control SNc neurons more strongly than SNr neurons; and 3) that chronic nicotine upregulated α4β2 nAChRs in both number and sensitivity so that it could augment the excitatory inputs to SNr from STN, indirectly inhibiting SNc neurons. Thus, STN neurons expressing α4β2 nAChRs might be a target for chronic nicotine, and confer neuroprotection.

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Graduate Students: Walter Bugg, Elaine Hsiao, Amber Southwell
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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 mice and over-expression *in vivo* with viral vectors, we are exploring the role of the neuropoietic cytokine leukemia inhibitor factor (LIF) in regulating neural stem cell proliferation and fate in the adult brain. In the context of neuroimmune interactions during fetal brain development, 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 (intrabodies) and also manipulating NFκB activity. We are also studying the regulation of MeCP2 by IKKα, because MeCP2 mutations are responsible for Rett syndrome, which frequently involves autism symptoms.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group contributed to the discovery of a family that we termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We 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 currently examining the role of LIF in a chemical model of multiple sclerosis, where exogenous LIF can increase oligodendrocyte number and stimulate remyelination.

Cytokine involvement in a model for mental illness is also being investigated. This mouse model is based on findings that maternal infection can significantly increase the likelihood of schizophrenia and 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. The cytokine IL-6, acting on both the placenta and fetal brain, is key in mediating the effects of maternal immune activation (MIA) on the fate of the offspring. We have new evidence that MIA alters the endogenous immune cells in the placenta, as well as lymphocyte reactions to stimulation in the adult offspring. In collaboration with the Mazmanian laboratory at Caltech, we are also examining the effects of MIA on gastrointestinal tract inflammation in the offspring.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin (Htt), the protein that causes HD. We produced single-chain intrabodies that bind to various domains of Htt, and these can either exacerbate or alleviate Htt toxicity in cultured cells, acute brain slices, and in *Drosophila* HD models. Recent findings indicate that viral delivery of one of these intrabodies in five different mouse models of HD is highly effective in ameliorating the behavioral deficits and neuropathology caused by mutant Htt in these models. 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. In collaboration with the Langen laboratory at USC, using electron paramagnetic resonance spectroscopy we have obtained new structural information on the domains of mutant Htt when it forms fibrils.

343. Identifying the sites of interleukin-6 action following maternal immune activation

Elaine Hsiao, Paul H. Patterson

Maternal infection increases the risk for schizophrenia and autism in the offspring. In rodents, maternal influenza infection or maternal immune activation (MIA) with the double-stranded RNA, poly(I:C) causes behavioral and histological changes in adult offspring that are consistent with those seen in schizophrenia and autism. This suggests 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, it was determined 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. In fact, injecting pregnant mice with recombinant IL-6 is sufficient to induce in offspring the development of behavior deficits that are equivalent, if not more severe, than those observed in poly(I:C) offspring. Therefore, localizing the sites of IL-6 action may illuminate the anatomical and molecular pathways through which MIA alters fetal brain development. Our data suggest that IL-6 acts on both the placenta and the fetal brain. Downstream IL-6 responses indicative of IL-6 activity, such as SOCS3 induction, are observed in the fetal brain and placenta following maternal poly(I:C) injection. Moreover, IL-6 signaling pathways are activated in the placenta and fetal brain shortly after maternal poly(I:C) injection, as measured by increased phosphorylation of STAT1 and STAT3. An IL-6-specific activation of STAT3 is localized to spongiotrophoblast cells in the placenta, while MIA-induced phosphorylation of STAT3 and STAT1 is localized to sinusoidal trophoblast giant cells in the labyrinth layer of the placenta. These effects indicate that MIA is transduced in the fetal compartment and suggest that MIA may alter the balance of uterine hormones and signaling factors that affect fetal development. Maternal poly(I:C) also increases IL-6 mRNA expression itself in the fetal brain and placenta, raising the possibility of a positive feedback loop. That these effects of poly(I:C) are due to IL-6 induction is indicated by an experiment in which an anti-IL-6 blocking antibody is co-injected with poly(I:C), which abrogates the observed increases in IL-6 and its downstream sequelae. These results support a key role for IL-6 in the placenta and fetal brain in shaping neural development and behavior in the MIA offspring.

344. The effect of maternal immune activation on the ultrasonic vocalizations of the neonatal 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. A behavioral assay that can be used early in development involves ultrasonic vocalizations (USVs), which are important for mother–infant social interaction. We find that i.p. injection of the double-stranded RNA, poly(I:C), in C57BL/6J pregnant females at mid-gestation alters pup USVs in the isolation test. Ten-day-old pups from immune-activated mothers display a lower rate of USVs compared to pups born to saline-injected mothers. In addition, analysis of sonogram structure shows differences in the repertoire of calls emitted by male pups. Compared to controls, pups from immune-activated mothers emit significantly fewer composite calls and more short calls. As adults, males from poly(I:C)-injected mothers display a deficit in a social interaction test in which they are given a choice between interacting with another mouse or spending time in an empty chamber. In addition, these males show significantly fewer USVs in response to a female mouse stimulus. In sum, these results suggest that maternal immune activation yields males with poor social and communicative behavior, which are hallmarks of autistic-like behavior. This is consistent with our finding that the offspring of immune-activated mothers also display a neuropathology that is frequently found in autism, a spatially-restricted deficit in Purkinje cells (Shi *et al*., 2009).

345. Imaging hallucinations in mice

Natalia Malkova, Paul H. Patterson

Hallucinations are defined as the normal activation of the visual or auditory system in the absence of appropriate sensory input. A corollary is that such activity should be enhanced by drugs that are known to induce hallucinations in normal people and that exacerbate this symptom in schizophrenic subjects. Activation of 5-HT2A receptors (5-HT2ARs) is responsible for the psychomimetic properties of hallucinogens in humans. 5-HT2AR agonists such as 2,5-dimethoxy-4-iodoamphetamine (DOI) and lysergic acid diethylamide (LSD) stimulate head twitches in mice, which are not seen in 5-HT2AR null mutant mice. We find that DOI induces this stereotyped behavior in mice in a dose-dependent manner. At the molecular level, DOI activates the immediate early genes *egr-1* and *c-fos* expression in the auditory, visual and somatosensory cortices. Thus, in the absence of external acoustic and visual input, the hallucinogen DOI activates surrogate markers of neuronal activity in the cortex.

The second stage of the project involves our mouse model (Shi *et al*., 2003) that is based on the epidemiological finding that maternal infection increases the risk of schizophrenia in the offspring. We find that, compared to controls, the offspring of mothers whose immune systems were activated at mid-gestation show increased stereotypical behavioral responses (head-twitching, grooming and rearing) to DOI. This increased sensitivity to the hallucinogen raises the question of whether they also experience spontaneous, hallucination-like, neuronal activity. We are exploring the use of novel functional MRI methods to examine this question.

346. Asymmetrical reduction of calcium binding protein expression in the MIA model

Jan Ko, Yael Piontkewitz, Ina Weiner, Paul H. Patterson

Epidemiological studies have linked maternal infections, both viral and bacterial, to higher risk of developing schizophrenia and autism in the adult offspring. Factors common to all infections are thought to be responsible for the adverse effect on fetal brain development. The synthetic double-stranded RNA poly I:C, which stimulates an antiviral inflammatory response without viral infection, is used to model maternal immune activation (MIA). Structural MRI reveals that adult MIA offspring have enlarged ventricles and smaller hippocampal volumes. These hallmarks of schizophrenia

are not apparent in adolescent offspring, however. In a therapeutic intervention, giving the anti-psychotic drug clozapine during adolescence prevents the onset of both structural and behavioral changes in MIA offspring. Another neuropathological feature of prefrontal cortex in schizophrenia is a deficit in immunostaining for the calcium binding proteins calbindin and parvalbumin in subpopulations of GABA interneurons. Likewise, we find that in female MIA offspring, parvalbumin staining is reduced in cortical layers and calbindin staining is reduced in hippocampal subfields. However, these deficits occur only one side of the brain. This asymmetry has also recently been reported in both a chemical and a genetic mouse model of autism. Because of the known role of these GABA interneurons, is possible that this asymmetry has effects on circuits underlying rhythmic patterns of brain activity.

347. Poly(I:C) mouse model of mental illness - Urine analysis and impact of diet

Catherine Bregere

Infections during pregnancy increase the risk of mental disorders such as schizophrenia and autism. It is the maternal immune activation, rather than the infectious agent itself that mediates abnormalities in the development of the fetal brain, and subsequent abnormal behavior. This environmental risk factor can be modeled in pregnant mice. Indeed, dams injected with poly(I:C), a viral mimic, yield progeny with abnormal neurodevelopment and behavior. To enhance our understanding of this mouse model at the molecular level, we sought to look for biomarkers in the urine of poly(I:C)-exposed progeny, using HPLC-electrochemical detection, a sensitive technique that allows quantitation of small molecules. Preliminary results indicate that serotonin and its metabolite 5-HIAA are significantly decreased in the urine of poly(I:C)-exposed male offspring in comparison to saline-exposed animals. This indicates that the peripheral serotonergic system is affected in the progeny of poly(I:C) treated dams, as urinary serotonin and 5-HIAA derives mainly from the gut. Additional small molecules, including tryptophan, will be quantified in saline and poly(I:C) exposed progeny. Another environmental factor under investigation is the impact of diet regimen on the maternal immune response. Epidemiologic data indicate that the risk of schizophrenia is higher for the progeny of mothers with high pre-pregnancy body mass index than that for mothers with lower body mass index. In this context, we hypothesized that a high fat regimen may affect the vulnerability to immune challenges. To test this hypothesis, mice are placed either on a low or high fat diet for 5 weeks, and then mated. Preliminary results indicate that the immune system of fat dams is impaired compared to lean dams, as evidenced by a significantly lower IL-6 response 3h following poly(I:C) administration in the former.

348. IKKβ regulates HD pathogenesis

Ali Khoshnan, Jan Ko

Proteolysis of huntingtin (Htt) plays a key role in the pathogenesis of Huntington's disease (HD). The N-terminal fragments generated from mutant Htt have the propensity to form oligomers and amyloid structures that are neurotoxic. We have identified IKKβ as a regulator of Htt cleavage and neurotoxicity, as inhibitors of IKKβ prevent Htt cleavage and are able to reduce neurotoxicity of N-terminal Htt fragments. IKKβ may also regulate neuroinflammation in HD. Presymptomatic HD patients produce elevated levels of proinflammatroy cytokines in the sera and CNS years before the onset of symptoms, and IKKβ is a prominent kinase that regulates cytokine production. Thus, IKKβ is likely to play a prominent role in HD pathogenesis. We are now in the process of examining the role of IKKβ in HD pathogenesis *in vivo.* Towards this aim, we have generated an HD mouse model that lacks IKKβ in the CNS. These mice will be examined for neuropathology, motor deficits and expression of inflammatory markers in CNS. We will also examine the effects of RNAi and small molecule inhibitors of IKKβ on HD pathology *in vivo.* These studies should determine whether IKKβ is a therapeutic target for HD.

349. IKKα promotes neuronal differentiation

Ali Khoshnan

IKKα is a serine/threonine kinase and a component of the IκB kinase complex (IKKα, IKKβ, and IKKγ), which regulates the NF-κB pathway. IKKα is also a chromatin-modifying enzyme. In cells stimulated by cytokines, IKKα enters the nucleus and is able to phosphorylate several substrates including histone-3, CREB binding protein (CBP), and the silencing mediator of retinoid and thyroid hormone receptor (SMRT). This leads to the assembly of an active chromatin configuration. Although IKKα is expressed in the CNS, its functions are not well understood. We have reported that IKKα promotes the survival of human neurons exposed to genotoxic stress. Thus, IKKα may influence neuronal gene expression in response to environmental stimuli and possess neuroprotective properties.

We are using human neuronal stem cells to examine whether changes in the homeostasis of the IKKs affects differentiation and lineage commitment. To enhance the nuclear function of IKKα, we introduced an extra copy into a human mesencephalic neuronal stem cell line (MESC2.10). Using this model, we have identified a novel function for IKKα in neuronal differentiation and maturation. We find that neuronal differentiation promotes nuclear localization of IKKα and induces the degradation of the neuron-restrictive silencer factor (NRSF), a prominent repressor of neuronal gene expression. Subsequently, production of neurogenic factors, including miRNA-124a, synapse associated proteins and MeCP2, is enhanced. IKKα also promotes the expression of BDNF, a growth factor associated with synaptic plasticity. Interestingly, the IKKα-induced

BDNF is independent of MeCP2 levels, which is known to regulate BDNF expression. Knockdown of MeCP2 expression has no major effects on BDNF levels, however. These findings prompted us to examine the mRNA profiles of differentiated neurons with elevated IKKα and their derivates with knockdown of MeCP2 expression in order to dissect the role of IKKα in mediating MeCP2-dependent and independent gene regulation. Using mRNA sequencing, we find hundreds of mRNAs that are significantly altered by IKKα over-expression. Some of the IKKα-regulated genes are also modulated by MeCP2. Detailed analysis of these studies may reveal the role of IKKα in neuronal differentiation and how its association with MeCP2 regulates chromatin structure.

350. Regulation of MeCP2 function by IKKα

Ali Khoshnan, James Li

Homeostasis of methyl-CpG binding protein 2 (MeCP2) is critical for neuronal function and development. Mutations in the coding region of MeCP2 are responsible for most cases of Rett syndrome. The levels of MeCP2 are also reduced in the brains of a subset of idiopathic autism patients. MeCP2 is expressed at high levels in mature neurons and it is suggested to play a role in dendritic growth and synaptic plasticity. MeCP2 is known to bind to methylated CpG islands in the promoters of many genes, where it recruits a repressor complex to inhibit gene expression. However, recent studies indicate that MeCP2 is also a transcriptional activator. MeCP2 may influence the expression of up to 2,200 genes, including BDNF and CREB (Chahrour *et al*., 2008). The context and environmental signals that regulate the transcriptional activity of MeCP2 are poorly understood. Phosphorylation of MeCP2 at several serine residues is important for its biological function. Activity-induced phosphorylation of MeCP2 at Ser 421 by calcium/calmodulin-dependent protein kinase II (CaMK-II) in neurons induces gene expression from several promoters including the exon-IV BDNF, enhances dendritic branching, and regulates complex behaviors such as seizures and circadian rhythm in animal models (Zhou *et al*., 2006). Ser 80 phosphorylation, on the other hand, is essential for association of MecP2 with chromatin. It is notable that mass spectrometric analysis of MeCP2 isolated from brain reveals the presence of several phosphorylated sites, some which have not been characterized (Tao *et al*., 2009). Thus, phosphorylation-mediated regulation of MeCP2 function remains an exciting area of investigation.

We have identified IκB kinase alpha (IKKα) as a potential modulator of MeCP2 expression and activity in neurons. We find that in a differentiating neuronal stem cell line elevated IKKα enhances the accumulation of MeCP2 and MeCP2 phosphorylated at Ser-421. IKKα binds to and phosphorylates MeCP2. Mass spectrometric analysis revealed Ser 326 as a novel phosphorylation site in MeCP2. We are exploring whether IKKα may phosphorylate at other sites as well. IKKα, along with MeCP2 and CREB, are recruited to the promoter of MeCP2-regulated genes such as the exon-IV BDNF promoter, and this appears to enhance promoter activity. We propose that IKKα and MeCP2 may form a molecular switch, which could influence chromatin structure in post-mitotic neurons and thus, affect MeCP2 targets such as BDNF. These studies have implications for the neurobiology of Rett syndrome and potentially other neuropsychiatric disorders where MeCP2 functions and its levels are altered. IKKα is responsive to cytokines and in many cases suppresses the production of inflammatory cytokines. Thus, a major goal of this project is to explore the consequences of IKKα/MecP2 interaction in response to environmental cues.

351. Domain organization of mutant Huntington fibrils

Charles W. Bugg, Paul H. Patterson, Ralf Langen[*]

A hallmark of HD is the formation of fibrillar aggregates within cells. *In vitro*, huntingtin exon 1 (HDx1) with a polyQ expansion forms fibrils that have a cross beta structure common to amyloid fibrils found in other diseases such as Alzheimers, Parkinsons and prion diseases. Little else is definitively known about HDx1 structure, however. We used electron paramagnetic resonance spectroscopy to study the organization of the major domains of mutant HDx1 within the fibril. We find that these fibrils do not have parallel in register structure, like most other disease-associated amyloid fibrils. The C-terminus is highly dynamic and is attached like a tail to the polyQ domain, which is mostly immobilized and forms the core of the fibril. However, the C-terminal portion of the polyQ tract lies outside the core of the fibril and has a mobility similar to the C-terminus. The N-terminus produced heterogeneous spectra, indicating that it is able to sample multiple conformations. In sum, our study excluded the parallel, in-register arrangement of beta strands within HDx1 fibrils and represents a first step towards a high-resolution structure of HDx1 fibrils.

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352. Exogenous LIF stimulates oligodendrocyte progenitor cell proliferation and remyelination

Benjamin E. Deverman

We have found that delivery of leukemia inhibitory factor (LIF) to the adult brain promotes neural stem cell self-renewal and stimulates oligodendrocyte progenitor cell (OPC) proliferation. Based on these data, we hypothesized that if exogenous LIF could enhance the OPC response in the context of chronic demyelination it may, in turn, promote the generation of new oligodendrocyte (OLs) and aid remyelination. To test this, we first fed mice a diet containing cuprizone, a treatment that induces a robust and reproducible course of demyelination in the corpus callosum (CC) and hippocampus, and then injected the mice with either a LIF- or lacZ-expressing adenovirus (Ad-LIF or Ad-lacZ) in the lateral ventricle. We find that LIF significantly enhances OPC proliferation in the CC and hippocampus. In the hippocampus,

where spontaneous generation of new OLs and remyelination is minimal after a 12-week course of cuprizone, the number of OLs is restored to near normal levels with 6 weeks of LIF treatment, while in Ad-lacZ-treated mice the number of OLs fails to recover. Furthermore, compared to controls, remyelination in the hippocampus is significantly greater in Ad-LIF-treated mice, and in these mice, nodes of Ranvier reform on a subset of remyelinated axons. Finally, we have initiated a set of experiments in which we are inducibly knocking out the gp130 subunit of the LIF receptor in various cell populations *in vivo*. First, we are deleting gp130 specifically in OPCs to investigate whether LIF stimulates the proliferation of OPCs directly via cell-autonomous gp130 signaling, or through factors produced by intermediate cell types. Preliminary findings suggest that LIF induces OPC proliferation in a gp130-dependent, cell-autonomous manner. In addition, we are using these mice to investigate whether LIF promotes OPC differentiation into mature OLs. Second, we will cross gp130 floxed mice with additional lines to test whether gp130 signaling is required for OL lineage specification, differentiation, and myelination during development, and whether gp130 signaling regulates survival and proliferation of OL lineage cells during demyelination. These studies will yield important insight into the effects that LIF and related gp130 cytokines have on OLs and their response to injury, and whether the effects of LIF can be harnessed to enhance remyelination.

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Summary: More than 10% of Americans suffer from chronic sleep disorders, with an estimated annual cost of $100 billion and for which therapeutic options are poor. Despite the impact of sleep disorders, the fact that we sleep for a third of our lives, and the evolutionary conservation of sleep-like behaviors, the mechanisms that regulate sleep remain poorly understood. It is therefore important to develop simple and cost-effective systems to study the genetic and neural regulation of sleep. Zebrafish are a useful system for these studies because: 1) unlike invertebrates, fish have the basic brain structures thought to regulate mammalian sleep; 2) larval zebrafish are transparent, which makes it easy to monitor and manipulate their neurons; and 3) zebrafish are amenable to high-throughput screens that can identify genes, drugs and neurons that regulate sleep. Zebrafish are therefore a useful system for unraveling the mysteries of sleep. The goal of our lab is to address two fundamental questions: What genetic and neural mechanisms regulate sleep?

353. A high-throughput screen for small molecules that affect zebrafish sleep

David A. Prober[#], Jason Rihel[#], Alexander F. Schier[*]*

A major obstacle for the discovery of psychoactive drugs is the inability to predict how small molecules will affect complex behaviors. We developed and applied a high-throughput, quantitative screen for small molecules that alter the behavior of larval zebrafish. We screened ~6000 bioactive small molecules and identified ~500 compounds that affect sleep/wake behaviors. The multidimensional nature of observed phenotypes allowed us to cluster molecules according to shared phenotypes. This approach revealed conserved functions of psychotropic molecules in regulating sleep/wake states and predicted the mechanisms of action of poorly characterized compounds. In addition, this screen implicated new factors in the control of sleep/wake behaviors, including ether-a-go-go-related gene potassium channels, L-type calcium channels, immunomodulators, and several compounds whose targets are unknown. This screen demonstrated the power of high-throughput behavioral profiling in zebrafish to discover and characterize psychotropic drugs and to dissect the pharmacology of complex behaviors. We are now characterizing some of the small molecule sleep regulators in more detail.

[#]*Equal contribution*
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354. A screen for genes that regulate sleep/wake behaviors

David A. Prober, Jason Rihel[*], Viveca Sapin, Alexander F. Schier[*]

The genetic regulation of sleep is poorly understood. Identifying genes that regulate sleep is therefore a high priority in sleep research. Genetic screens in *Drosophila* have begun to identify genes that regulate invertebrate sleep-like states, but such screens have not been performed in vertebrates. To identify genes that regulate vertebrate sleep, we developed a novel approach to inducibly overexpress genes that encode secreted proteins and assay effects on sleep/wake behaviors in a high-throughput manner. We screened ~1000 genes, and identified ~30 genes that affect specific sleep/wake behaviors. We are currently generating stable transgenic lines for each gene to verify results from the screen and to perform more detailed behavioral analyses. Because few vertebrate genes are known to regulate sleep, these studies have the potential to transform our understanding the genetic regulation of sleep.

[*]*Harvard University*

355. Analysis of zebrafish Hypocretin neural circuit architecture and activity

Cindy Chiu, Adam Kampff[*], Eva Naumann[*], Florian Engert[*], Alexander F. Schier[*], David A. Prober

Loss of Hypocretin (Hcrt) or Hcrt-expressing neurons causes the sleep disorder narcolepsy, which affects ~1 in 2000 humans and is characterized by excessive daytime sleepiness, fragmented sleep/wake states and cataplexy. We, and others, have shown that the Hcrt system is anatomically and functionally conserved in zebrafish. However, while Hcrt is the best characterized vertebrate sleep regulator, it is not fully understood how the Hcrt system regulates sleep. This goal is difficult to achieve in mammals, which possess thousands of Hcrt neurons that project widely throughout the brain. In contrast, zebrafish larvae only have ~10 Hcrt neurons, providing a simpler system to study the development and basic functions of these neurons. To determine how zebrafish Hcrt neurons regulate sleep, we are characterizing the development, activity and function of these neurons at the single neuron level using three approaches. First, we are using the Brainbow technique to label each Hcrt neuron with a different color and map out the projections of each Hcrt neuron. Second, we developed a bioluminescence-based approach to monitor the activity of genetically specified neurons in freely behaving zebrafish larvae. Using this approach, we found that zebrafish Hcrt neurons are active during wakefulness and silent during rest. We are now testing whether the correlation between Hcrt neuron activity and wakefulness requires Hcrt function and analyzing the relationship between the activities of individual Hcrt neurons and sleep/wake behaviors. Third, we will use optogenetic techniques to determine the effects of stimulating and inhibiting Hcrt neurons on behavior.

[*]*Harvard University*

356. The role of adenosine in regulating zebrafish sleep/wake behaviors

Avni Gandhi, David A. Prober

Adenosine is thought to regulate sleep in mammals, but little known about its role in regulating sleep in other animals. Furthermore, mammalian studies have provided conflicting results regarding which adenosine receptors are involved in sleep, and the neural substrates of adenosine function in sleep are controversial. Using a small molecule approach, we found evidence that adenosine signaling promotes sleep in zebrafish larvae. We are now using genetic and pharmacological approaches to test the hypothesis that adenosine promotes sleep in zebrafish and to identify the relevant receptors. We will also determine where adenosine acts to regulate sleep and test the hypothesis, based on mammalian studies, that adenosine regulates sleep by modulating the Hcrt system.

357. The role of Prokineticin 2 in linking circadian cues to sleep/wake states

Shijia Chen, David A. Prober

Circadian signals ensure that sleep occurs during the appropriate time of the 24 hour circadian cycle, but the neural and genetic mechanisms that link circadian cues to sleep are poorly understood. A candidate molecule that may convey circadian signals to behavior is Prokineticin 2 (Prok2). This peptide is secreted by the mammalian suprachiasmatic nucleus (SCN) in a circadian manner and inhibits locomotor activity when overexpressed at night, when rodents are primarily awake. We isolated the zebrafish Prok2 ortholog and found that it is expressed in the larval zebrafish presumptive SCN. Furthermore, overexpression of Prok2 inhibits locomotor activity during the day, when zebrafish larvae are most active. We are now characterizing the expression pattern and behavioral function of Prok2 in larval zebrafish in more detail. We are also attempting to isolate the Prok2 promoter to study the development and function of Prok2-expressing neurons.

358. Identification of factors that regulate Hypocretin neuron specification

Justin Liu, Florian Merkle[*], Alexander F. Schier, David A. Prober*

The mechanisms that specify hypothalamic neural populations are poorly understood. We are studying the zebrafish orthologs of the neuropeptides Hypocretin (Hcrt) and QRFP, which regulate feeding, metabolism and wakefulness in mammals, as models to explore mechanisms that regulate the specification of hypothalamic neural populations. As in mammals, *hcrt*- and *qrfp*-expressing neurons are located adjacent to each other in the embryonic zebrafish hypothalamus, but they are never coexpressed in the same cell. We used *hcrt*-mRFP; *qrfp*-EGFP transgenic zebrafish and flow cytometry to isolate pure populations of *hcrt*- and *qrfp*-expressing neurons when they are first specified in zebrafish embryos. We then performed microarray analyses to identify genes whose expression is enriched in Hcrt and QRFP neurons compared to each other and compared to all neurons. These experiments identified ~10 transcription factors whose expression is enriched in Hcrt or QRFP neurons. We hypothesize that some of these transcription factors are necessary and/or sufficient for Hcrt or QRFP neuron specification. We are currently testing this hypothesis using gain and loss of function approaches.

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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 extended the ERATO (Exploratory Research for Advanced Technology) Shimojo "Implicit Brain Functions" project (supported by JST, Japan Science and Technology Corporation, October '04 – March '10), into the new CREST (Core Research for Evolutional Science and Technology, started in April '10) project on "InterPersonal Implicit Communication" (supported also by JST) with its emphasis on implicit cognitive processes, emotional decision making, social communication, and their neural correlates. Vigorous collaborations are conducted among our psychophysics laboratory here and the CREST Japan site located at NTT Communication Science Laboratories and Tamagawa University Brain Science Institute in Japan, as well as Harvard MGH, Boston University, Gordon College London, National Academy of Science Austria, Occidental College, and Decode Inc., Germany. Besides, we continue collaborative efforts on "social brain," under the Caltech-Tamagawa gCOE (grand Center Of Excellence) program (supported by MEXT, Ministry of Education, Culture, Sports, Science and Technology, Japan, started in Sept '08).

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 CREST and gCOE projects, we aim to understand implicit, as opposed to explicit or conscious, somatic and neural processes that lead to, and thus predict, conscious emotional decision such as preference. As a more overarching general theme including both the perceptual psychophysics and the CREST/gCOE 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. The new gCOE program put additional scientific emphases on social, emotional, and reward-related neural circuits. Toward these ends, we employ interpersonal EEG recording, TMS/tDCS stimulation, and fMRI-base neural feedback method.

In extensions of our core interest in visual preference decision making, we collaborated with neuroeconomists on campus to investigate neural correlates of choice decision in more realistic real-world situations (such as purchasing snacks).

(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. On top of our earlier finding of TMS-triggered "visual replay" effect (see the annual report last year), we had found that after repeated paired presentation of the visual transient stimulus and the dual-pulse TMS (say ten trials or more), the TMS alone can trigger the replay without visual stimulation ("entrainment effect"). This paired association effect was sensitive to the temporal delay between the visual stimulus and TMS in the pairing phase. Including the original and the entrainment version of it, the main issue had been as to whether this replay is perceptually "real" or is it some sort of cognitive artifact, nothing like a reactivation of the real visual circuits in the brain. By applying a newly-devised masking paradigm, we finally found firm evidence that the replay is *not* just a "phenomenological illusion" but rather executing real effects on visual information encoding. This is now successfully applied to the case of entrainment, as well as the original replay.

In an independent set of study, we compared the subjective timing of a TMS-triggered phosphene vs. the dual-pulse TMS itself (which triggers the phosphene). The results indicate a postdictive tendency of perception, i.e., "re-writing" of the sequence of events in a very brief time period in the visual cortex.

(2) Emotional decision making has been the central focus of our ERATO and CREST projects. We have investigated behavioral and neural correlates of such preference decision in both the human and the monkey. In particular, we addressed the issue of memory-preference relationship, in order to resolve the seeming conflict between the Familiarity and the Novelty principles in the literature. We originally found a surprised segregation between these two principles across object categories - the Familiarity dominates across trials in face preference, whereas, the Novelty dominates in natural scene preference. With lots of additional control results

indicating robustness of the finding, the paper is finally published (PNAS, '10).

Our subsequent studies have explored how attractiveness of image components are integrated in complex images, such as an object (face or geometric figure) surrounded by other faces, or hair attached to a face, etc. The overall patterns of result can be summarized in a relatively simple way, as the following: (a) When the task was to evaluate attractiveness of the whole image, a simple, quasi-linear model holds, that is, attractiveness of components merely sum up; and (b) When the task was to evaluate attractiveness of an attended object (say, hair) while neglecting the other (face), a more complicated non-linear interaction takes place. Depending on the perceptual connection between the attended vs. the neglected, there can be a positive leakage of attractiveness from the neglected to the attended, or a negative leakage (i.e., a negative contrast).

In yet another study, we presented two faces in sequence, and investigate neural responses to each by EEG, to find predictive components to the preference decision in multivariate EEG signals. Our results indicate three distinctive EEG components, including event-related-potential (ERP) and wavelet-based time-frequency-representations (TFR), from the posterior to the frontal parts of the brain.

(3) We conducted several behavioral and/or fMRI studies all related to reward-related decision making, whose results can be summarized as the following: (a) In a simplified poker game, a poker face turned out not to be the best poker face, but instead, a trust-worthy face is it, because the opponent tended to fold more; (b) We found a common representation of decision values for dissimilar goods, such as foods and commercial products, in the human ventromedial prefrontal cortex; and (c) Likewise, we found that medial orbitofrontal cortex (mOFC) is engaged both for real and imagined rewards.

(4) In addition, we have conducted a couple of seed-seeking studies in different directions, one in sensory-motor aspects of the feeling of "agency," the other in postdictive or hindsight tendency in perception and memory. The results were promising in each, suggesting that: (a) the feeling of self-control ("agency") can be disrupted by a motor program erroneously triggered, as indicated in the "escalator illusion;" (b) Apparent motion is supported by a postdicitive interpolation mechanism; and (c) there is a massive tendency towards postdiction in people's episodic memory on own prediction.

(5) In a following up analysis on the MEG responses to color flicker stimulus in the neurotypical and atypical (photo epileptic) subjects, we found that the disruption of correlations in the MEG responses is the indicator of pathological changes.

359. Retrieval of visual percept by paired association of a visual stimulus and transcranial magnetic stimulation (TMS): Objective evidence from a masking paradigm

Hsin-I Liao, Neil Halelamien, Daw-An Wu, Shinsuke Shimojo

TMS to visual cortex interacts with retinal input, altering perceptual experience. When double-pulse TMS is applied following a visual stimulus, an "instant replay" of the percept could be perceived. Furthermore, after repeating several pairs of the visual stimulus and TMS, the replay percept could be retrieved by TMS alone without accompanying with visual stimulus (Liao *et al*., ASSC '08; Liao and Shimojo, APCV '08). Here, we adopted a masking paradigm developed by Vasudevan *et al.* (VSS '09) to examine whether the retrieved percept causes similar masking effect, as well as the replay. A pattern mask was paired with TMS to produce a spatially and temporally specific replay masking effect on a letter identification task. After ten or five trials, TMS followed by the target letter with identical delay is delivered without the pattern mask. In results, the replay of pattern mask was retrieved by TMS alone to degrade the identification performance. When the pattern mask appeared at a different location from the letter, no masking was observed for replay or retrieved percept. That both the replay and the retrieved percept caused location-specific masking provides objective evidence for a common perceptual mechanism underlying the replay and the retrieval.

360. Perceived timing of TMS-induced phosphene

Junghyun Park, Shinsuke Shimojo

The visual pathway has a considerable afferent delay. It takes anywhere between 40-100 ms for the neural signal of a visual stimulus to reach to V1. It may take even longer to have a substantial population of neurons to be activated to encode the visual event. On the other hand, transcranial magnetic stimulation (TMS) induces visual percepts (phosphenes) by directly affecting neuronal activity in visual cortex, bypassing the time-consuming afferent pathway from retina to visual cortex. This, however, will not necessarily guarantee that phosphene is perceived earlier than an externally presented stimulus, considering the unnatural activity profile that TMS may invoke in the visual cortex.

To see if indeed the TMS-induced phosphene is perceived substantially earlier than the retinal input, in Experiment 1, the subjects performed a temporal order judgment (TOJ) test between an external light flash (a white bar) and phosphene. The results of the TOJ test showed that on average the perceptual latency of the phosphene was 94 ms shorter than that of the bar.

In Experiment 1, TMS was applied by a pair of Magstim 200 monophasic pulse generators connected to a figure-of-eight coil via a Magstim Bistim unit. Most of subjects could not perceive phosphenes with single monophasic pulse even with a high TMS output intensity. But when a pair of monophasic pulses was delivered with a 30 ms inter-pulse interval, most subjects clearly saw

phosphenes at moderate output intensities, suggesting a spatio-temporal summation of dual-pulse stimulation. This raises another question: what would be the timing of phosphene perception - the time of the first pulse or the second?

To answer this question, in Experiment 2, subjects performed a series of TOJ tests between the bar and phosphene where the inter-pulse interval of the dual-pulse TMS was systematically varied (20-100 ms). The correlation between the points of subjective simultaneity (PSSs) and the inter-pulse intervals should be 1 (or 0), if the second (or the first) pulse is synchronized with the timing of phosphene perception. The results showed that the correlation between PSSs and inter-pulse intervals was on average far less than 1. This suggests that the perceived timing of the dual-pulse induced phosphene is referred backwards in time toward the time of first pulse, and provides a nice experimental tool to study the phenomenon of subjective backward referral.

361. Roles of familiarity and novelty in visual preference judgments are segregated across object categories

Junghyun Park, Eiko Shimojo, Shinsuke Shimojo

Understanding preference decision making is a challenging problem because the underlying process is often implicit and dependent on context, including past experience. There is evidence for both familiarity and novelty as critical factors for preference in adults and infants. To resolve this puzzling contradiction, we examined the cumulative effects of visual exposure in different object categories, including faces, natural scenes, and geometric figures, in a two-alternative preference task.

The results show a clear segregation of preference across object categories, with familiarity preference dominant in faces and novelty preference dominant in natural scenes. No strong bias was observed in geometric figures. The effects were replicated even when images were converted to line drawings, inverted, or presented only briefly, and also when spatial frequency and contour distribution were controlled. The effects of exposure were reset by a blank of 1 wk or 3 wk.

Thus, the category-specific segregation of familiarity and novelty preferences is based on quick visual categorization and cannot be caused by the difference in low-level visual features between object categories. Instead, it could be due either to different biological significances/attractiveness criteria across these categories, or to some other factors, such as differences in within-category variance and adaptive tuning of the perceptual system.

362. Visual attractiveness is leaky (1): center and surround

Eiko Shimojo, Chihiro Saegusa, Junghyun Park, Alexandra Souverneva, Shinsuke Shimojo

Repeated experience with a stimulus form a memory that affects preference decision in future. We have demonstrated that N(ovelty)/F(amiliarity) of a surrounding natural scene (NS) affects attractiveness of a central face (FC), even when the subjects neglected the surround NS (Shimojo, *et al*., VSS '09).

To examine further how N and F interact between a center (task-relevant) stimulus and surrounding (task-irrelevant) stimuli, we prepared three new stimulus sets in which a central FC(, NS, or GF) is surrounded by four others (always in the same object category), and N/F of the center and the surround were manipulated independently. According to pre-ratings, the baseline attractiveness was matched between the center and the surround. The subjects performed two tasks in separate sessions: (1) to rate attractiveness of the central stimulus *only*, or (2) to rate attractiveness of the *whole* image. Eye movements were recorded (by EyeLink 2).

The eye tracking results ensured the effectiveness of task instructions. Even when the subject focused on the attractiveness of the center only (in the task (1) above), it was implicitly affected by that of the surround modulated via memory. For example, attractiveness of the central new GF changed more positively across trials when the surround GF is new as opposed to old, which was however, not true for the central old GF. More in general, there are significant interactions between the central (new/old) and the peripheral (new/old) conditions.

Different factors, including: (a) segregation of N/F across object categories (Shimojo, *et al*., VSS '07); (b) modulation of N/F due to task-dependent attention; and (c) implicit contagion of attractiveness from outside of attention, will be considered.

363. Visual attractiveness is leaky (2): hair and face

Chihiro Saegusa, Eiko Shimojo, Junghyun Park, Shinsuke Shimojo

Memory-based attractiveness integration is implicit and nonlinear, as we demonstrated with images featuring a central face (FC) and a surrounding natural scene (NS) (Shimojo *et al*., VSS'09). Here, we aimed to see how the task-irrelevant surround affects attractiveness of the central stimulus and vice versa, using hair (HR) and face (FC). There is evidence that HR is indeed a surrounding, accessory part of the holistic FC perception (Ellis *et al*., 1980), and both are processed in the face-specific temporal area (Kanwisher *et al*., 1997).

Eight FC images (4 attractive and 4 less attractive FCs), and 16 HR pictures (4 colors, 2 lengths and 2 shapes) were selected from a pre-rated set. Each FC and HR were combined in the natural spatial alignment, and subjects were asked to rate attractiveness of 1) FC only or 2) HR only in a 7-point scale in separate sessions.

Results of 1) show that, when FC is shown with an attractive HR, the attractiveness of FC was higher than with a less attractive HR, even though subject was asked to focus only on the FC. Results of 2) were symmetrical to those of 1) in that the task-irrelevant FC affects attractiveness of HR. The overall patterns of the results cannot be simply interpreted as the subjects neglecting the "ONLY" instruction, because the "FC only" attractiveness

with HR is exceedingly lower than the range predicted from weighted averaging of the pre-rated attractiveness of HR and FC.

These results seem difficult to interpret unless we accept two possibilities: (a) the attractiveness of the task-irrelevant surround is implicitly "imported" into that of the central stimulus; and (b) something more nonlinear than just averaging occurs particularly in the FC only evaluation with HR.

364. Neural components underlying subjective preferential decision making

Job P. Lindsen, Rhiannon Jones, Shinsuke Shimojo, Joydeep Bhattacharya

The objectives of the current study were twofold: (i) to investigate the neural precursors of the formation of a subjective preference of facial stimuli, and (ii) to characterize the spatiotemporal brain activity patterns distinguishing between preferred and non-preferred faces. Multivariate EEG signals were recorded while participants made preference decisions, based on approachability, between two faces presented sequentially with unrestricted viewing time; the decision being made after presentation of the second face. The paired faces were similar in their physical properties, emphasizing the role of the subjective experience of the participants in making the decisions. EEG signals were analyzed in terms of event-related-potential (ERP) components and wavelet-based time-frequency-representations (TFR). The behavioural data showed that the presentation order and the exposure duration did not influence preference formation.

The EEG data showed three effects. The earliest effect, the sustained posterior ERP positivity for preferred first faces as compared to non-preferred first faces, was found following the onset of the first face, and this was interpreted as the formation of a positive first impression of the first face. The two later effects following the second faces were an increase of frontal theta band oscillations around 500 ms for preferred second faces and of posterior gamma band oscillations around 650 ms for preferred first faces; both of which were interpreted as being related to the formation of a preference.

All of these effects occurred well before the moment of conscious decision, thereby suggesting the implicitness of these neurally identifiable components.

365. Human wagering behavior depends on opponents' faces

Erik J. Schlicht, Shinsuke Shimojo, Colin F. Camerer, Peter Battaglia, Ken Nakayama

Research in competitive games has exclusively focused on how opponent models are developed through previous outcomes and how peoples' decisions relate to normative predictions. Little is known about how rapid impressions of opponents operate and influence behavior in competitive economic situations, although such subjective impressions have been shown to influence cooperative decision-making.

This study investigates whether an opponent's face influences players' wagering decisions in a zero-sum game with hidden information. Participants made risky choices in a simplified poker task while being presented opponents whose faces differentially correlated with subjective impressions of trust.

Surprisingly, we find that threatening face information has little influence on wagering behavior, but faces relaying positive emotional characteristics impact peoples' decisions. Thus, people took significantly longer and made more mistakes against emotionally positive opponents. Differences in reaction times and percent correct were greatest around the optimal decision boundary, indicating that face information is predominantly used when making decisions during medium-value gambles. Mistakes against emotionally positive opponents resulted from increased folding rates, suggesting that participants may have believed that these opponents were betting with hands of greater value than other opponents.

According to these results, the best "poker face" for bluffing may not be a neutral face, but rather a face that contains emotional correlates of trustworthiness. Moreover, it suggests that rapid impressions of an opponent play an important role in competitive games, especially when people have little or no experience with an opponent.

366. Evidence for a common representation of decision values for dissimilar goods in human ventromedial prefrontal cortex

Vikram S. Chib, Antonio Rangel, Shinsuke Shimojo, John P. O'Doherty

To make economic choices between goods, the brain needs to compute representations of their values. A great deal of research has been performed to determine the neural correlates of value representations in the human brain. However, it is still unknown whether there exists a region of the brain that commonly encodes decision values for different types of goods, or if, in contrast, the values of different types of goods are represented in distinct brain regions.

We addressed this question by scanning subjects with functional magnetic resonance imaging while they made real purchasing decisions among different categories of goods (food, nonfood consumables, and monetary gambles). We found activity in a key brain region previously implicated in encoding goal-values: the ventromedial prefrontal cortex (vmPFC) was correlated with the subjects' value for each category of good. Moreover, we found a single area in vmPFC to be correlated with the subjects' valuations for all categories of goods.

Our results provide evidence that the brain encodes a "common currency" that allows for a shared valuation for different categories of goods.

367. Human medial orbitofrontal cortex is recruited during experience of imagined, as well as real rewards

Signe Bray, Shinsuke Shimojo, John P O'Doherty

Human decision-making frequently relies on mental simulation 24 of future rewards in order to guide action choice. In this study, we sought to uncover brain regions engaged during reward imagery, and to address whether these regions functionally overlap with regions activated by tangible rewards.

We found that medial orbitofrontal cortex (mOFC) is engaged both for real and imagined rewards, and is preferentially engaged for imagery with rewarding content compared to other non-rewarding imagery. These findings support a critical role for mOFC in the representation of rewarding goal-states, even if hypothetical.

368. Odd sensation induced by moving-phantom that triggers subconscious motor program

Takao Fukui, Toshitaka Kimura, Koji Kadota, Shinsuke Shimojo, Hiroaki Gomi

Our motor actions are sometimes not properly performed despite our having complete understanding of the environmental situation with a suitable action intention. In most cases, insufficient skill for motor control can explain the improper performance. A notable exception is the action of stepping onto a stopped escalator, which causes clumsy movements accompanied by an odd sensation. Previous studies have examined short-term sensorimotor adaptations to treadmills and moving sleds, but the relationship between the odd sensation and behavioral properties in a real stopped-escalator situation has never been examined. Understanding this unique action-perception linkage would help us to assess the brain function connecting automatic motor controls and the conscious awareness of action.

Here we directly pose a question: Does the odd sensation emerge because of the unfamiliar motor behavior itself toward the irregular step-height of a stopped escalator or as a consequence of an automatic habitual motor program cued by the escalator itself. We compared the properties of motor behavior toward a stopped escalator (SE) with those toward moving escalator and toward a wooden stairs (WS) that mimicked the stopped escalator, and analyzed the subjective feeling of the odd sensation in the SE and WS conditions. The results show that moving escalator-specific motor actions emerged after participants had stepped onto the stopped escalator despite their full awareness that it was stopped, as if the motor behavior was guided by a "phantom" of a moving escalator. Additionally, statistical analysis reveals that postural forward sway that occurred after the stepping action is directly linked with the odd sensation. The results suggest a dissociation between conscious awareness and subconscious motor control: the former makes us perfectly aware of the current environmental situation, but the latter automatically emerges as a result of highly habituated visual input no matter how unsuitable the motor control is. This dissociation appears to yield an attribution conflict, resulting in the odd sensation.

369. Perception of apparent motion relies on postdictive interpolation

Zoltan Nadasdy, Shinsuke Shimojo

Ever since Wertheimer discovered apparent motion (AM), controversy about its mechanism (i.e., interpolation vs. extrapolation, postdictive vs. predictive) still lingers. In this series of experiments, we addressed both questions by presenting subjects an AM stimulus starting from the middle of the screen (phase 1) and terminating at either left or right (phase 2) unpredictably.

The subjects perceived both motions effortlessly, regardless of the apparent direction. Thus, the motion illusion must have been constructed in the brain only after phase 2, which determined the direction of motion. In the same experiment, we also flashed two targets simultaneously, during phase 2, at various spatial locations and asked subjects to report the temporal order of these targets. We found that almost all subjects perceived the two targets sequentially, between the two AM phases in time, when they were flashed between the AM stimuli. No sequential effect was detected on targets outside of the AM trajectory. These results are consistent with the interpolation hypothesis.

In a second experiment, we studied the dependency of sequential effect on different spatiotemporal configurations of targets. We introduced a marker to help subjects to disambiguate the order of intermediate targets and asked them to judge the co-occurrence of the marker with either target while the target configuration was varied. We applied two types of AM sequences, a "predictive" when targets were presented before the AM, and a "postdictive" when targets were presented after the AM. According to the results, the marker helped subjects to perceive the correct temporal order under the predictive condition but not under the postdictive condition.

We concluded that apparent motion perception is postdictive, that it relies on interpolation, and that the postdictive interpolation has a sequential masking/delaying effect on the perception of intermediate targets. The neuronal mechanism of this masking is yet to be determined.

370. Postdictive mental revision of expectation of success in sports

Koji Kadota, Motoo Okumura, Shinsuke Shimojo

People tend to "rewrite" their memory of their postdiction (the "sixth sense") and the result at various occasions in business, purchasing, gambling, sports, etc., in order to be more consistent with the causality framework (postdiction, or hindsight). To see how extensive and strong this cognitive tendency is even under situations where one is committed to the event and the memory of prediction is explicitly consolidated, we conducted a very simple survey in amateur top-level athletes.

High school and college teams in Volleyball, Basketball, and Kendo (Japanese traditional fencing), were selected, and each team member were asked to fill out a short questionnaire twice; once in the morning of the game/match, and second after it as soon as possible (typically in the evening of the same day). The two sets of questionnaire were very similar, consisting of questions with 7-point scale about their physical and psychological conditions, their relationship to teammates, etc. The key question was as to how well (s)he predict(ed) to do in the game/match. Thus, the same question was asked to the same athlete twice, once in a purely predictive form, second in a "postdiction of prediction" form.

The results show that they tended to rewrite their memory to be more consistent with the results of the game/match (i.e., win or lose). The results were symmetrical, both ways – that is, they tended to raise their prediction in their memory in case where they won, while to lower in case of lost. The results strongly indicate the overwhelming power of postdictive re-editing at a cognitive level, at mostly sub-conscious level even when the event is important and the memory of prediction is relatively fresh and explicit.

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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.

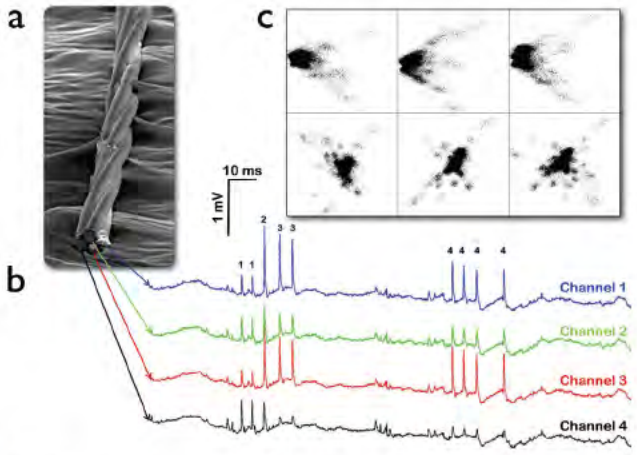

Figure 1: *Hippocampal Tetrode Recordings.* (a) Scanning EM image of a tetrode, which consists of four microwires twisted together to form a single recording probe. (b) Because of the spatial separation between the wire tips and the signal sources, each action potential is recorded simultaneously on 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.

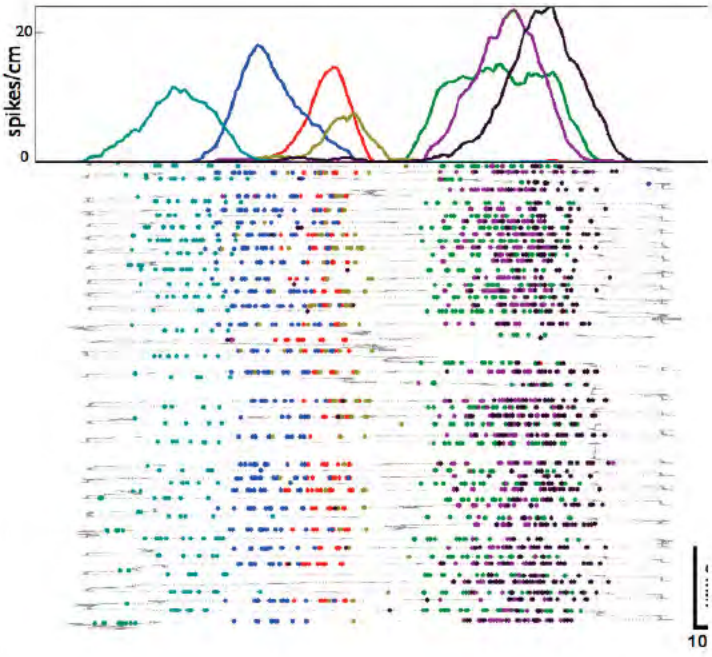

Figure 2: *Place fields.* The bottom panel shows that position of a rat in gray as a function of time (total time 28 minutes). The rat runs on a linear track back and forth and the positions and times of the spikes of seven simultaneously recorded place cells are shown in different colors. The top panel 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.

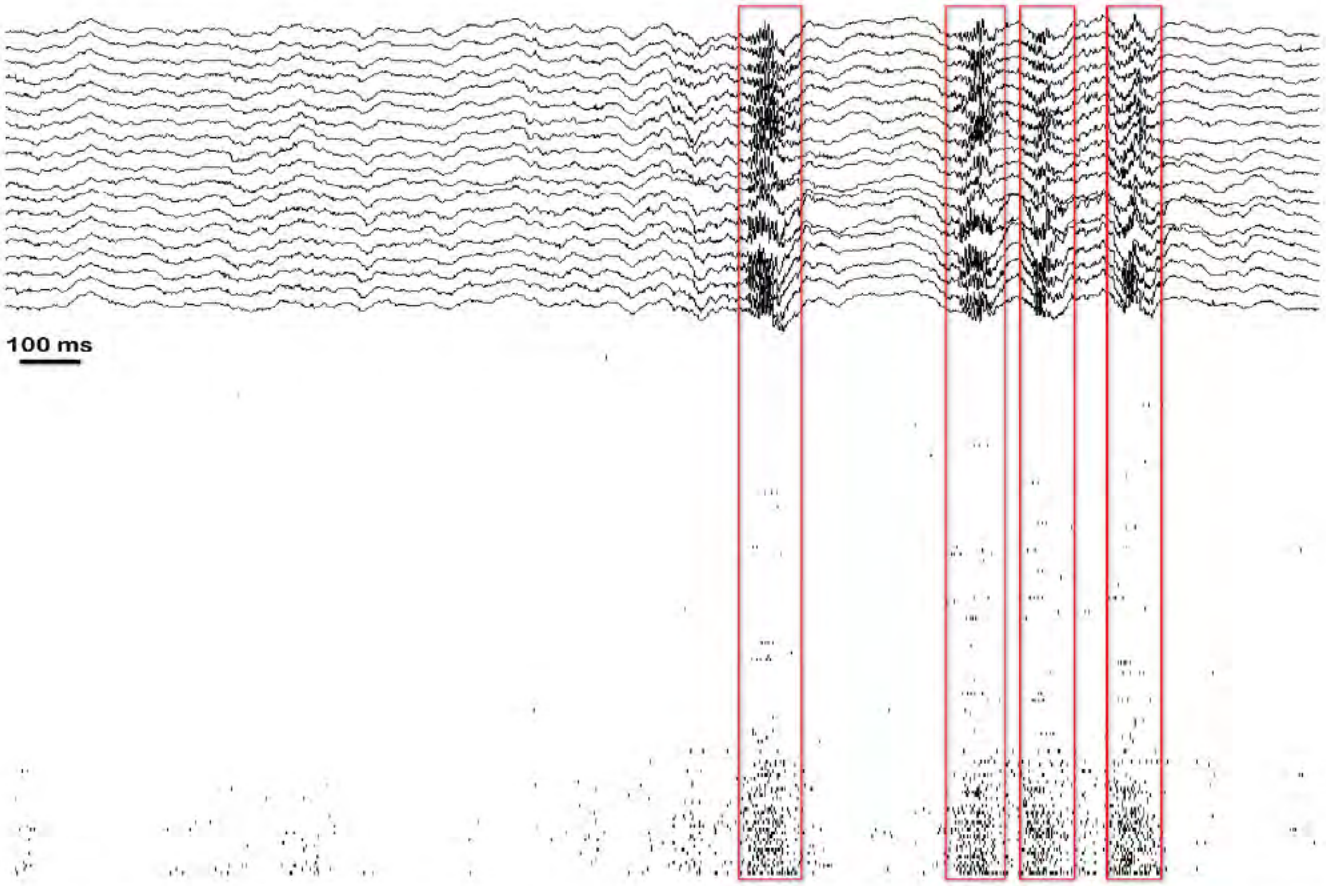

Figure 3: *Hippocampal synchronous bursts during slow-wave sleep.* Local field potential (LFP) recordings from several tetrodes in CA1 (top), together with rasters of 154 simultaneously recorded CA1 cells during slow-wave sleep. Rasters are ordered in terms of firing rates. A block of high firing interneurons 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.

371. Hippocampal theta oscillations are traveling waves

Evgueniy Lubenov, Thanos Siapas

Theta oscillations clock hippocampal activity during awake behaviour and rapid eye movement (REM) sleep. These oscillations are prominent in the local field potential, and they also reflect the subthreshold membrane potential and strongly modulate the spiking of hippocampal neurons. The prevailing view has been that theta oscillations are synchronized throughout the hippocampus, despite the lack of conclusive experimental evidence. In contrast, here we have shown that in freely behaving rats, theta oscillations in area CA1 are travelling waves that propagate roughly along the septotemporal axis of the hippocampus. Furthermore, we found that spiking in the CA1 pyramidal cell layer is modulated in a consistent travelling wave pattern. Our results demonstrate that theta oscillations pattern hippocampal activity not only in time, but also across anatomical space. The presence of travelling waves indicates that the instantaneous output of the hippocampus is topographically organized and represents a segment, rather than a point, of physical space.

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372. Decoupling through synchrony in recurrent networks

Evgueniy 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

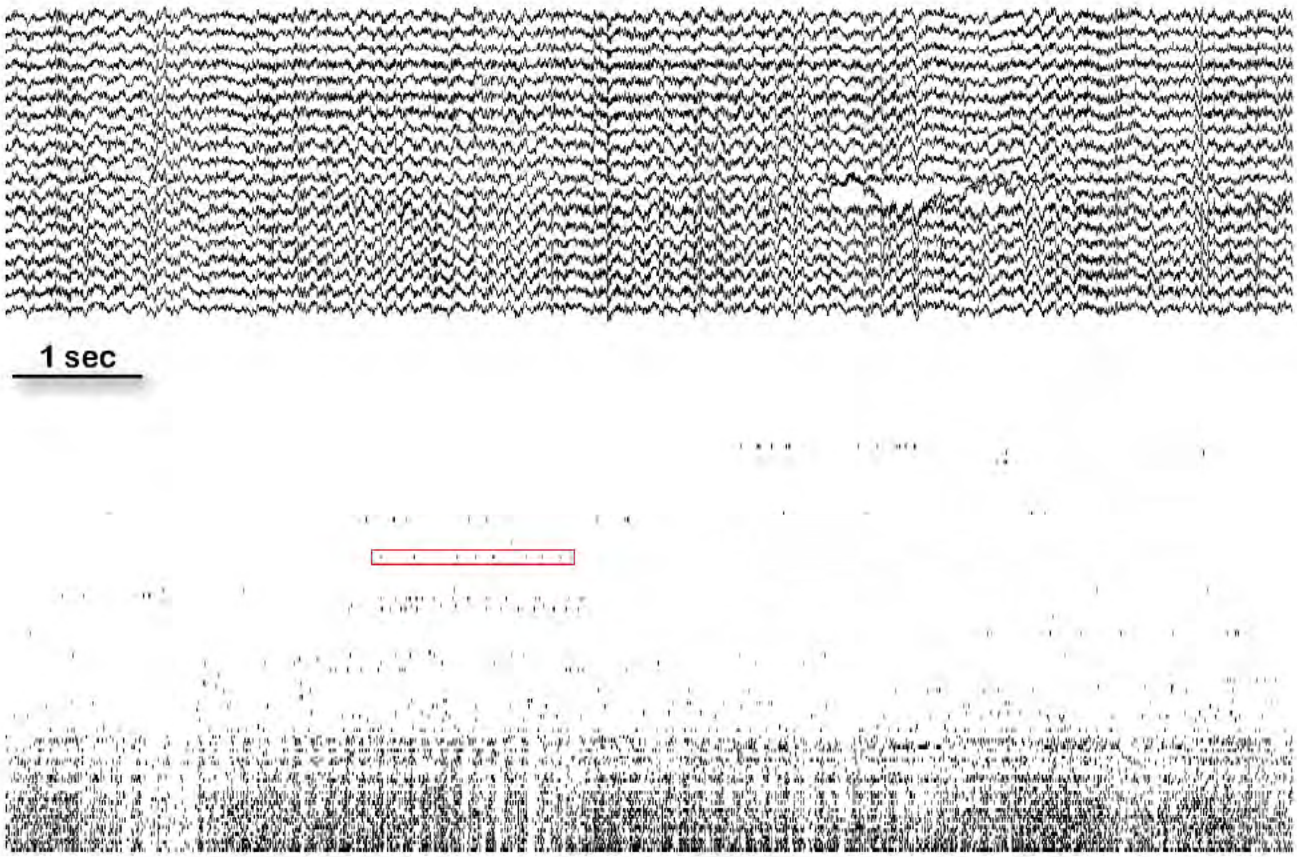

Figure 4: *Hippocampal activity during REM sleep.* The same neurons and LFP traces as in Figure 3 during a REM episode (2 minutes earlier than in Figure 3). Notice the scale change from Figure 3. Theta oscillations (4-10 Hz) are clearly visible in the LFPs, and interneurons fire rhythmically phase-locked to the theta oscillations.

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.

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373. Mechanisms and functional consequences of synchronous hippocampal bursts during slow-wave sleep

Evgueniy Lubenov, Casimir Wierzynski, 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.

374. Hippocampal activity patterns during REM sleep

Evgueniy Lubenov, 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

hippocampal activity across multiple sleep-wake cycles. In addition, we develop tools for analyzing and identifying network activity motifs in REM sleep and study their experience specificity.

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375. Prefrontal-hippocampal interactions during slow-wave sleep

Casimir Wierzynski, Evgueniy Lubenov, Ming Gu, Thanos Siapas

Cortico-hippocampal interactions during sleep are believed to reorganize neural circuits in support of memory consolidation. However, spike-timing relationships across cortico-hippocampal networks - key determinants of synaptic changes - are poorly understood. We have shown that cells in prefrontal cortex fire consistently within 100 ms after hippocampal cells in naturally sleeping animals. This provides evidence at the single cell-pair level for highly consistent directional interactions between these areas within the window of plasticity. Moreover, these interactions are state dependent: they are driven by hippocampal sharp-wave/ripple (SWR) bursts in slow-wave sleep (SWS) and are sharply reduced during REM sleep. Finally, prefrontal responses are nonlinear: as the strength of hippocampal bursts rises, short-latency prefrontal responses are augmented by increased spindle band activity and a secondary peak approximately 100 ms later. These findings suggest that SWR events are atomic units of hippocampal-prefrontal communication during SWS and that the coupling between these areas is highly attenuated during REM sleep.

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Summary: Research in our lab aims to uncover the mechanisms for visual perception in primates, specifically, how objects are identified, and how 3D shape and location are computed. We use a combination of fMRI, electrophysiology, and anatomy in monkeys, as well as mathematical modeling. In order to elucidate the principles of information flow underlying object recognition, we are investigating a specialized system of fMRI-identified face-selective regions in the temporal and frontal lobes of the macaque brain. A major goal is to understand the functional specialization of each of these face patches. To this end, we are interrogating the face system from four directions:

1. *Representations*: What are the selectivity and invariance properties of cells in each face patch?
2. *Behavioral Role*: What is the effect of inactivating specific face patches on various face-related behaviors?
3. *Connectivity:* What is the wiring diagram of the face patches?
4. *Transformations*: What are the key functional differences between input and output cells within each face patch?

We believe that understanding in detail the processing in the face patches will not only yield insights into the general mechanisms underlying object recognition, but will clarify basic organizational principles of primate neocortex.

In the coming years, we plan to follow a similar approach to understanding the neural mechanisms underlying spatial perception.

376. Faces in Motion: Mapping macaque and human face processing networks with dynamic stimuli

Pablo Polosecki, Simon Kornblith, Sebastian Moeller, Doris Y. Tsao, Winrich A. Freiwald[1,2]

Imaging and electrophysiological studies have revealed the existence of a system of brain areas in the macaque monkey and in humans specialized for face recognition. This system is composed of face-selective areas or patches in the temporal and prefrontal lobes. These areas have been localized by contrasting activity in response to faces with activity in response to a number of non-face objects, typically using static images. However, the use of more naturalistic, dynamic stimuli would be expected to provide stronger responses throughout the network. This approach may also reveal functional differentiation within the face-processing network (Hoffman and Haxby, 2000; Yovel and Kanwisher, 2005) and allow for a better understanding of potentially homologous face areas in macaques and humans.

Here, we report results obtained in fMRI experiments, in which we presented moving and static faces as well as moving and static control objects, performed in macaque monkeys and human subjects on a Siemens Tim TRIO scanner during passive fixation. In Monkeys, contrast agent MION was used. We found slightly enhanced responses to moving faces as compared to static faces throughout the face-processing network. However, in humans, one of the areas, the STS face area, showed a marked increase in response magnitude to moving compared to static faces. In macaque monkeys, we also observed a general increase of activity to moving compared to static faces and, in two face patches, MF and AF, a marked increase to moving faces. These results show the existence of face areas tuned to dynamic stimuli; demonstrate the potential of identifying putative homologies between face areas of two species as different as humans and macaque monkeys; and underscore the importance of the use of more naturalistic, behaviorally relevant stimuli for mapping the face processing network.

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377. Representations of multiple objects in the macaque face processing system

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Primates excel in recognizing faces in cluttered scenes. It has even been proposed that faces "pop out" of arrays of objects (1). Face recognition is thought to be mediated by specialized face processing areas. In the macaque monkey, six bilateral face-selective regions have been found in the temporal lobe by functional magnetic resonance imaging (fMRI). These regions, termed face patches, consist of one posterior, two middle and three anterior face patches, named after their anatomical location along the temporal lobe. They are structurally connected to form a dedicated face-processing network. Anatomical location and functional properties of neurons in different face patches suggest a hierarchical arrangement of face patches, along which both invariance and selectivity properties increase. Remarkably, even at an early processing level of the system, many neurons have large receptive fields (of more than ten degrees diameter), and face selectivity is maintained even at the largest eccentricities. Thus, in natural environments these

receptive fields will most often encompass multiple stimuli simultaneously.

Here we investigated the question of how multiple stimuli are represented by neurons in the middle face patches. Face patches were localized by fMRI and targeted with recording electrodes, guided by structural MRI. We recorded responses to a range of stimuli including individual faces and objects at different locations of the receptive field, and combinations of these stimuli. This allowed us to determine stimulus selectivity and response latency in different parts of the receptive field and test whether the response to multiple stimuli approaches the average or the maximum of the responses to the individual stimuli (2). We found that simple models of response combination could not account for the responses to multiple stimuli we observed. First, the magnitude of the response to stimulus pairs did not follow directly from the responses to individually presented stimuli. Rather spatial location within the receptive field turned out to be strong determinant of response magnitude. Second, the temporal profile of the response to stimulus pairs could exhibit a dynamics not anticipated by responses to individual stimuli. We present recording results from the middle face patches and discuss them in the context of current models of hierarchical shape processing in inferotemporal cortex.

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378. Neural substrates of face perception awareness in the rhesus monkey

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Face perception is the ability to identify and recognize faces quickly and effortlessly independently of changes in illumination, size, occlusion, etc. Both macaques and humans have a dedicated system for processing faces in the temporal lobe. In the macaque, this system is composed of small patches of cortex in which almost all the cells are selective for faces.

To study the correlates of subjective face awareness, we used continuous flash suppression to render stimuli invisible: a target (a face or an object) was presented to one eye while a flashing mask was presented to the other eye. We measured the psychophysical visibility of the target in human subjects as a function of mask contrast, and determined two conditions, one of complete visibility and one of complete invisibility.

Two rhesus monkeys were scanned with MION contrast agent in a Siemens TIM Trio scanner while performing a simple passive fixation task. Blocks of visible faces, visible objects, invisible faces, invisible objects, binocularly visible low contrast faces, and binocularly visible high contrast faces, were presented.

The activation map obtained by contrasting visible faces versus visible objects revealed six temporal face patches bilaterally, consistent with results using a standard face localizer. The contrast of invisible faces versus invisible objects did not activate any areas in the brain. Specifically, all six temporal face patches showed strong activation to visible faces and no activation to invisible faces above that to invisible objects. In contrast, binocularly visible low contrast faces elicited significant activation above that to binocularly visible low contrast objects in all the face patches. These results suggest that activity within the face patch system correlates with subjective awareness of perceived faces, as far back as the most posterior face patch, PL, located in area TEO.

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379. Representation of faces in AM

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The macaque temporal lobe contains six patches of face-selective cortex. We have previously shown that these regions are tightly inter-connected (1) and subserve distinct functions (2). Furthermore, we have shown that the most anterior temporal patch (AM, located anterio-lateral of the AMTS) projects to face-selective regions in prefrontal cortex (3), as well as to the amygdala and claustrum (1). Therefore, AM may contain the final output of temporal lobe face processing.

We identified the six temporal face patches in monkeys in an fMRI experiment contrasting faces versus non-face objects and scrambled images. We then used tungsten electrodes to record from AM. During recording animals performed a simple fixation task while images were presented centrally.

To characterize the selectivity of AM neurons, we tested cells with two sets of stimuli. The first set ("localizer") consisted of 50 faces each at three different views and 50 nonface objects. The second set consisted of 425 frontal views of human faces, 8 frontal views of macaque faces, and 50 nonface objects. Cells in AM were highly face selective. Multi-dimensional-scaling analysis revealed that species and facial complexion constituted two important dimensions of AM face representation. In particular we found 19 of 89 cells were highly selective for monkey faces. Furthermore, AM cells represented faces in a view-invariant way as a population, and this view-invariant representation did not appear to require extensive familiarization with different views of the same individual. AM cell responses recorded in the first week of exposure to the localizer stimuli already encoded a view-invariant representation. These results suggest that AM does not map face space uniformly but is biased to represent ethologically relevant stimuli (e.g., conspecifics). In addition, the templates underlying view-invariant face recognition in AM may largely be hard-wired.

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380. Contrast tuning in face cells – evidence for region based encoding

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Several state of the art computer vision systems for face detection, e.g., Viola-Jones [1], rely on region-based features that compute contrast by adding and subtracting average image intensity at specific locations within a face. This is a powerful strategy due to the invariance of these features across changes in illumination (as proposed by Sinha [2]). The computational mechanisms underlying face detection in biological systems, however, remain unclear. We set to investigate the role of region-based features in the macaque middle face patch, an area that consists of face-selective neurons. We presented a parameterized face where each part contained unique intensity level, allowing us to determine the tuning for contrast across different face parts. First, we found that fully inverting the contrast across all face parts reduced firing rate by 50% on average. We then analyzed the sensitivity of cells to the contrast relationship between specific pairs of parts within a face. We found that individual neurons were tuned to subsets of contrast relationships between pairs of face parts. The sign of tuning for these relationships was strikingly consistent across the population (for example, almost all neurons preferred a lower average intensity in the eye region relative to the nose region). Furthermore, the pairs and polarity of the tuning were fully consistent with Sinha's proposed ratio-template model of face detection [2]. Non-face images from the CBCL dataset that contained correct contrast polarities in pre-defined regions (facial parts) did not elicit increased firing in face-selective neurons, suggesting that neurons are not only computing averaged intensity according to a fixed template, but are also sensitive to the specific shape features within a region.

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381. A place area in the macaque temporal lobe

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In humans, functional magnetic resonance imaging has identified an area in posterior parahippocampal cortex that exhibits significantly stronger activation to images of scenes than to images of faces or objects. In three macaques, we localized a potentially homologous area bilaterally in the fundus of the occipitotemporal sulcus that responded significantly more strongly to images of both empty and furnished familiar and novel indoor scenes than to images of scrambled scenes, familiar and unfamiliar objects, textures, and scenes in which the background was blurred but the objects were left intact. In line with previous human findings (Epstein and Kanwisher, 1998), the region exhibited a reduced response to images of scenes in which the walls and floor were rearranged such that they no longer formed a coherent geometry. This region potentially serves a role in visual guidance of spatial navigation.

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Summary: The fruit fly *Drosophila melanogaster* is our primary experimental system. We work on a variety of problems, mostly concerning the molecular mechanisms by which synaptic connections are formed during development. 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. We have also begun a new project to study the mechanisms involved in defining the geometries of tubes in the tracheal (respiratory) system. Our approaches combine genetics, molecular biology, electrophysiology, biochemistry, and cell biology. The current group members include five postdocs: Robert Carrillo, Peter Hyung-Kook Lee, Kaushiki Menon, Ed Silverman, and Ashley Wright; 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). Another postdoc (Mili Jeon) is continuing her work on RPTPs in Matthew Scott's lab at Stanford. Bader Al-Anzi, a postdoc from the Benzer lab, is also associated with our group, and Elena Armand works with him.

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 976 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 410 of the 976 CSS genes in our database, representing about 40% of the repertoire and including members of all CSS protein families. This database has since been employed for a variety of projects.

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 30 genes that cause synaptic mistargeting on muscles 12 and 13, and 55 genes that cause synaptic morphology phenotypes.

We focused initially on the analysis of the mistargeting genes, as this is our primary interest. In a paper published in 2008 in *Neuron* (Kurusu *et al.*) we showed that one class of receptor, the leucine-rich repeat (LRR) family, is overrepresented within the mistargeting set. Using a combination of loss-of-function and gain-of-function analysis, we demonstrate that four of these LRR proteins participate in the decision of the RP5 motor axon to choose muscle 12 as its target. These proteins are: Tartan (TRNA), Capricious (Caps), CG14351/Hattifattener (Have), and CG8561/Als (Kurusu/Cording abstract).

Since publication of this paper, Amy Cording has continued to evaluate other members of the LRR family for potential roles in axon guidance and synapse formation. We have also collaborated with Liwung Lou's group at Stanford, who used our collection of lines to identify proteins involved formation of synaptic connections in the antennal lobe. This work has led to the publication of a paper showing that Caps and TRNA are also involved in targeting of projection neuron dendrites to specific glomeruli of the antennal lobe (see Publication list).

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. The patterns of redundancy are summarized in a paper (Jeon *et al.*) published in 2008.

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 (Df) mutations that remove specific portions of the genome.

Using the Df 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). Sdc is a heparan sulfate proteoglycan (HSPG) that binds to Lar with nanomolar affinity and participates in its axon guidance functions. This work was published in 2005. We have continued the Df screen, and have identified three regions required for 99A-AP staining, and identified the corresponding genes. Analysis of these indicates 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). We began with the Bloomington Df kit, but have replaced many of the lines with other Dfs for which homozygotes have more normal development. 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. These results have been recently published (see Wright abstract).

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 and Zinn (2005), 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. Peter Lee has already found many such lines, and these define more than 20 new RPTP ligands.

Tracheal development: Regulation of EGFR tyrosine kinase activity by the Ptp10D and Ptp4E RPTPs regulates lumen formation.

In the process of examination of double mutants lacking expression of the closely related proteins Ptp4E and Ptp10D, we noticed that that the tracheal network exhibits a unique phenotype in these embryos. Unicellular and seamless tracheal tubes develop bubble-like cysts in place of tubular lumen. The double mutation affects the sculpting of lumen into a tubular shape, a process that has not been studied before using molecular genetics. Cyst formation is partially due to hyperactivation of the Egfr tyrosine kinase, and Ptp10D and Egfr directly associate in cultured cells. We have published a paper describing this work (see Jeon abstract).

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 studied 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), GluRIIB is downregulated, while the alternative subunit GluRIIB is upregulated. Thus, the phenotypes of nos and pum are opposite in this system. We also show that Pum represses Nos expression. Regulation of GluRIIA and Nos by Pum involves direct binding of Pum to the 3' UTRs of their mRNAs. A paper describing this work was recently published (Menon *et al*.).

Assembly of Pumilio into ordered aggregates as a regulatory switching mechanism.

We are also studying Pum in another context: its potential role as 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* forms amyloid fibrils *in vitro*. 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 recently published a paper describing these findings (Salazar/Silverman/Menon/Zinn abstract).

382. Systematic screening of *Drosophila* deficiency mutations for embryonic phenotypes and orphan receptor ligands

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We describe collections of *Drosophila* deletion mutations (deficiencies) that can be systematically screened for embryonic phenotypes, orphan receptor ligands, and genes affecting protein expression and localization. Deficiency 'kits' that cover the genome with a minimum number of lines have been established to facilitate gene mapping. These kits cannot be systematically analyzed for phenotypes, however, since embryos homozygous for many deficiencies in these kits fail to develop due to the loss of key gene products encoded within the deficiency. To create new kits that can be screened for phenotype, we examined the development of the nervous system in embryos homozygous for more than 700 distinct deficiency mutations. Using this information, we defined a kit of ~400 deficiency lines, encompassing >80% of the genome, for which homozygotes have a recognizable nervous system and intact body walls. Here we show examples of screens of this kit for orphan receptor ligands and neuronal antigen

expression. It can also be used to find genes involved in expression, patterning, and subcellular localization of any protein that can be visualized by antibody staining. A subset kit of 233 deficiency lines, for which homozygotes develop relatively normally to late stage 16, covers ~50% of the genome. Screens of this kit have revealed new axon guidance phenotypes in the central nervous system and neuromuscular system and permitted a quantitative assessment of the number of potential genes involved in regulating guidance of specific motor axon branches. The subset kit could be employed to screen for phenotypes affecting all embryonic organs. In the future, these deficiency kits will allow *Drosophila* researchers to rapidly and efficiently execute genome-wide anatomical screens that require examination of individual embryos at high magnification.

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383. Regulation of synaptic Pumilio function by an aggregation-prone domain

Anna M. Salazar, Edward J. Silverman, Kaushiki P. Menon, Kai Zinn

We identified Pumilio (Pum), a *Drosophila* translational repressor, in a computational search for metazoan proteins whose activities might be regulated by assembly into ordered aggregates. The search algorithm was based on evolutionary sequence conservation patterns observed for yeast prion proteins, which contain aggregation-prone glutamine/asparagine (Q/N)-rich domains attached to functional domains of normal amino acid composition. We examined aggregation of Pum and its nematode ortholog PUF-9 by expression in yeast. A domain of Pum containing the Q/N-rich sequence, denoted as NQ1, the entire Pum N-terminus, and the complete PUF-9 protein localize to macroscopic aggregates (foci) in yeast. NQ1 and PUF-9 can generate the yeast *Pin+* trait, which is transmitted by a heritable aggregate. NQ1 also assembles into amyloid fibrils *in vitro*. In *Drosophila*, Pum regulates postsynaptic translation at neuromuscular junctions (NMJs). To assess whether NQ1 affects synaptic Pum activity *in vivo*, we expressed it in muscles. We found that it negatively regulates endogenous Pum, producing gene dosage-dependent *pum* loss-of-function NMJ phenotypes. NQ1 coexpression also suppresses lethality and NMJ phenotypes caused by overexpression of Pum in muscles. The Q/N block of NQ1 is required for these phenotypic effects. Negative regulation of Pum by NQ1 might be explained by formation of inactive aggregates, but we have been unable to demonstrate that NQ1 aggregates in *Drosophila*. NQ1 could also regulate Pum by a "dominant-negative" effect, in which it would block Q/N-mediated interactions of Pum with itself or with cofactors required for translational repression.

384. Live dissection of *Drosophila* embryos: streamlined methods for screening mutant collections by antibody staining

Hyung-Kook (Peter) Lee, Ashley Wright

Drosophila embryos between stages 14 and 17 of embryonic development can be readily dissected to generate "fillet" preparations. In these preparations, the central nervous system runs down the middle, and is flanked by the body walls. Many different phenotypes have been examined using such preparations. In most cases, the fillets were generated by dissection of antibody-stained fixed whole-mount embryos. These "fixed dissections" have some disadvantages, however. They are time-consuming to execute, and it is difficult to sort mutant (GFP-negative) embryos from stocks in which mutations are maintained over GFP balancer chromosomes. Since 2002, our group has been conducting deficiency and ectopic expression screens to identify ligands for orphan receptors. In order to do this, we developed streamlined protocols for live embryo dissection and antibody staining of collections containing hundreds of balanced lines. We have concluded that it is considerably more efficient to examine phenotypes in large collections of stocks by live dissection than by fixed dissection. Using the protocol described here, a single trained individual can screen up to 10 lines per day for phenotypes, examining 4-7 mutant embryos from each line under a compound microscope. This allows the identification of mutations conferring subtle, low-penetrance phenotypes, since up to 70 hemisegments per line are scored at high magnification with a 40X water-immersion lens.

385. Receptor tyrosine phosphatases control tracheal tube geometries through negative regulation of EGFR signaling

Mili Jeon, Kai Zinn

The formation of epithelial tubes with defined shapes and sizes is essential for organ development. Here we describe a unique tracheal tubulogenesis phenotype caused by loss of both *Drosophila* type III receptor tyrosine phosphatases (RPTPs), Ptp4E and Ptp10D. Ptp4E is the only widely expressed *Drosophila* RPTP, and is the last of the six fly RPTPs to be genetically characterized. We recently isolated mutations in *Ptp4E*, and discovered that, although *Ptp4E* null mutants have no detectable phenotypes, double mutants lacking both Ptp4E and Ptp10D display synthetic lethality at hatching due to respiratory failure. In these double mutants, unicellular and terminal tracheal branches develop large bubble-like cysts that selectively incorporate apical cell surface markers. Cysts in unicellular branches are enlargements of lumen that are sealed by adherens junctions, while cysts in terminal branches are cytoplasmic vacuoles. Cyst size and number are increased by tracheal expression of activated Egfr tyrosine kinase, and decreased by reducing Egfr levels. Ptp10D forms a complex with Egfr in transfected cells. Downregulation of EGFR signaling by the RPTPs is required for construction of tubular lumens, whether extracellular or intracellular, by cells that undergo

remodelling during branch morphogenesis. The *Ptp4E Ptp10D* phenotype represents the first evidence of an essential role for RPTPs in epithelial organ development. These findings may be relevant to organ development and disease in mammals, because DEP-1 (PTPRJ), an ortholog of Ptp4E/Ptp10D, interacts with the hepatocyte growth factor receptor tyrosine kinase. PTPRJ corresponds to the murine *Scc1* (suppressor of colon cancer) gene.

We have also shown that Rho family GTPases are relevant to the phenotype. Expression of dominant-negative Rho1 or Rac1 in tracheae suppresses the phenotype, while expression of constitutively activated Rho1 enhances it. This enhancement, however, is distinct from that produced by activated Egfr, because 'soap-bubble'-like networks of cysts form on ventral unicellular branches. These are not seen when activated Egfr is coexpressed. Also, activated Egfr causes cysts to appear on the dorsal branch, while activated Rho1 does not.

386. The leucokinin pathway and its neurons regulate meal size in *Drosophila*

Bader Al-Anzi, Elena Armand, Paul Nagamei, Margaret Olszewski[*], Viveca Sapin, Christopher Waters, Kai Zinn, Robert J. Wyman[*], Seymour Benzer

Total food intake is a function of meal size and meal frequency, and adjustments to these parameters allow animals to maintain a stable energy balance in changing environmental conditions. The physiological mechanisms that regulate meal size have been studied in blowflies, but have not been previously examined in *Drosophila*.

Here we show that mutations in the *leucokinin neuropeptide (leuc)* and *leucokinin receptor (lkr)* genes cause phenotypes in which *Drosophila* adults have an increase in meal size and a compensatory reduction in meal frequency. Since mutant flies take larger but fewer meals, their caloric intake is the same as that of wild-type flies. The expression patterns of the *leuc* and *lkr* genes identify small groups of brain neurons that regulate this behavior. Leuc-containing presynaptic terminals are found close to Lkr neurons in the brain and ventral ganglia, suggesting that they deliver Leuc peptide to these neurons. Lkr neurons innervate the foregut. Flies in which Leuc or Lkr neurons are ablated have defects identical to those of leucokinin pathway mutants.

Our data suggest that the increase in meal size in *leuc* and *lkr* mutants is due to a meal termination defect, perhaps arising from impaired communication of gut distension signals to the brain. Leucokinin and the leucokinin receptor are homologous to vertebrate tachykinin and its receptor, and injection of tachykinins reduces food consumption. Our results suggest that the roles of the tachykinin system in regulating food intake might be evolutionarily conserved between insects and vertebrates.

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387. Colorimetric measurement of triglycerides cannot provide an accurate measure of stored fat content in *Drosophila*

Bader Al-Anzi, Kai Zinn

Drosophila melanogaster has recently emerged as a useful model system in which to study the genetic basis of regulation of fat storage. One of the most frequently used methods for evaluating the levels of stored fat (triglycerides) in flies is a coupled colorimetric assay available as a kit from several manufacturers. This is an aqueous-based enzymatic assay that is normally used for measurement of mammalian serum triglycerides, which are present in soluble lipoprotein complexes. In this short communication, we show that coupled colorimetric assay kits cannot accurately measure stored triglycerides in *Drosophila*. First, they fail to give accurate readings when tested on insoluble triglyceride mixtures with compositions like that of stored fat, or on fat extracted from flies with organic solvents. This is probably due to an inability of the lipase used in the kits to efficiently cleave off the glycerol head group from fat molecules in insoluble samples. Second, the measured final products of the kits are quinoneimines, which absorb visible light in the same wavelength range as *Drosophila* eye pigments. Thus, when extracts from crushed flies are assayed, much of the measured signal is actually due to eye pigments. Finally, the lipoprotein lipases used in colorimetric assays also cleave non-fat glycerides. The glycerol backbones liberated from all classes of glycerides are measured through the remaining reactions in the assay. As a consequence, when these assay kits are used to evaluate tissue extracts, the observed signal actually represents the amount of free glycerols together with all types of glycerides. For these reasons, findings obtained through use of coupled colorimetric assays on *Drosophila* samples must be interpreted with caution. We also show here that using thin-layer chromatography to measure stored triglycerides in flies eliminates all of these problems.

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Summary: Neuroscientists study each of the 100 billion neurons in the human brain. But while they understand individual neurons, they've been stumped by how neurons work together, how they encode information, and how they generate thoughts, emotions, and actions. That pioneering area of study is behind the Broad Fellows Program in Brain Circuitry, made possible through an $8.9 million grant from the Broad Foundations and philanthropist Eli Broad in 2005.

Recipients of the Broad Fellowship in Brain Circuitry are able to devote up to five years to their projects, without having to worry about finding another postdoctoral appointment in a year or two, or limiting themselves only to research that will lead to tenure. These researchers are at a level between postdoctoral fellow and assistant professor, which means that they are very independent and don't have to worry about the tenure clock. The program is designed to give researchers the freedom and flexibility to advance their work in whatever way is most productive, and may include the development of specific technologies or the invention of new instruments. Broad Fellows are given individual space to do their work in the Beckman Laboratories of Behavioral Biology. The program strongly encourages independent cutting-edge research and provides commensurate funding, space and facilities for Fellows.

The program is funded by a generous gift from the Eli and Edythe Broad Foundation, a Los Angeles-based venture philanthropy focused on entrepreneurship for the public good in education, science, and the arts. Further funding is provided by various federal and private institutions that support the research of individual fellows. The program is overseen and directed by Prof. Christof Koch. Currently, five Fellows are in residence. Their research programs are described below.

388. Developing viral vectors for targeting vertebrate and invertebrate neurons

S. Cassenaer

We are broadly interested in issues related to neural coding and memory formation. We seek to address questions such as how stimuli from the outside world are represented by patterns of neural activity; what kind of information processing is carried out by successive and embedded neural circuits; and how patterns are stored, recalled, and integrated with new information by networks of neurons.

These issues are ideally investigated by methods that can precisely but minimally invasively manipulate and measure neuronal activity, while simultaneously monitoring the animal's behavior as it responds to well-controlled stimuli. Genetically encoded molecular tools that permit the perturbation and measurement by optical means can contribute significantly to this effort, provided that they can be targeted specifically to defined neuronal populations. Our work consists of the development of viral vectors to carry out such targeting, as well as their characterization and use in electrophysiological experiments.

The evaluation of the specificity of targeting vectors is carried out *in vitro*, in stable cell lines and in primary cultures of dissociated neurons, as well as *in vivo*. When determined to be sufficiently specific, viral vectors are used in conjunction with electrophysiological measurements in awake behaving animals

389. Measuring neurophysiological correlates of behavior in mice with silicon microelectrodes

J. Du, K. Gunapala, H.A. Lester, A.D. Steele, S.C. Masmanidis

Miniaturization of neural recording devices via micro and nanofabrication is essential to the development of minimally invasive neural prosthetics that interface with the central nervous system. Moreover, scaling down of probe dimensions is important for systems level studies of the brain, where high density electrophysiological measurements can greatly contribute to the understanding of coupling within and between cortical regions. Silicon remains the substrate material of choice for a variety of micromachined tools. Until now, the use of silicon probes in the mouse brain has been largely overlooked, possibly because of additional limitations imposed by the mouse's small weight and size. Nevertheless, many advanced and elegant genetic manipulations in mammals are only feasible in mice, presenting several unique opportunities for studying the neuronal substrates of behavior. Such studies would greatly benefit from the ability to carry out large-scale neuronal recordings in freely moving animals. We developed high density silicon-based microelectrode arrays and chronically implanted them in mice. Extracellular neuronal measurements were carried out in tandem with acquisition of behavioral information via video recording and annotation. The performance of the probes was characterized in several animals using chronic impedance and signal measurements, as well as

immunofluorescence imaging of tissue surrounding probes implanted for up to eight weeks. The number of single-neurons that could reliably be detected was found to decrease after approximately the first ten days *in vivo*. We speculate this effect is due to the chronic inflammatory response of the surrounding tissue. The silicon microelectrodes are currently being used in studies of: (i) short and long-loop feedback in the midbrain dopamine pathway, and (ii) neuronal substrates of food anticipatory activity.

390. Palatable meal anticipation in mice

C. Hsu, D. Patton[*], R. Mistlberger[*], A.D. Steele

The ability to sense time and anticipate future events is a critical skill in nature. Most efforts to understand the neural and molecular mechanisms of anticipatory behavior in rodents have used daily restricted food access, which induces a robust increase of locomotor activity in anticipation of daily meal time. Interestingly, rats also show increased activity in anticipation of a daily palatable meal even when they have an ample food supply, suggesting a role for brain reward systems in anticipatory behavior, and providing an alternate model by which to study the neurobiology of anticipation in species, such as mice, that are less well adapted to "stuff and starve" feeding schedules. To extend this model to mice, and exploit molecular genetic resources available for that species, we tested the ability of wild-type mice to anticipate a daily palatable meal. We observed that mice with free access to regular chow and limited access to highly palatable snacks of chocolate or "fruit crunchies" avidly consumed the snack but did not show anticipatory locomotor activity as measured by running wheels or video based behavioral analysis. However, mice receiving a small meal of high fat chow did show increased food bin entry prior to access time and a modest increase in activity in the two hours preceding their scheduled meal. These results indicate that anticipation of a food reward in mice is behavior and diet specific, and may be limited to food resources that are especially high in fat calories.

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391. Automated home-cage behavioral phenotyping of mice

H. Jhuang[*], E. Garrote[*], X. Yu, V. Khilnani, T. Poggio[*], A.D. Steele, T. Serre[*]

We describe a trainable computer vision system enabling the automated analysis of complex mouse behaviors. We provide software and a very large manually annotated video database used for training and testing the system. Our system performs on par with human scoring, as measured from ground-truth manual annotations of thousands of clips of freely behaving mice. As a validation of the system, we characterized the home-cage behaviors of two standard inbred and two non-standard mouse strains. From this data we were able to predict in a blind test the strain identity of individual animals with high accuracy. Our video-based software will complement existing sensor based automated approaches and enable an adaptable, comprehensive, high-throughput, fine-grained, automated analysis of mouse behavior.

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392. Daily timed social interaction induces moderate anticipatory activity in mice

C. Hsu, D. Chang, P. Dollar, A.D. Steele

How an animal's brain can be programmed to anticipate future events is an unanswered question. Rodents display robust anticipatory activity in the hours preceding timed daily access to food when access is limited to a short temporal duration. We tested whether this anticipatory behavior could be generalized to timed daily social interaction by examining if singly housed male mice could anticipate either a daily novel female or a familiar female. We observed that anticipatory activity was moderate under both conditions, although both a novel female partner and sexual experience are moderate contributing factors to increasing anticipatory activity. Restricted access to running wheels did not produce any anticipatory activity, suggesting that an increase in activity during the scheduled access time was not sufficient to induce anticipation. To tease apart social versus sexual interaction, we tested the effect of exposing singly housed female mice to a companion female mouse daily for one hour for 28 days. The female mice did not show anticipatory activity for restricted female access, despite a large amount of social interaction, suggesting that daily timed social interaction between mice of the same gender is insufficient to induce anticipatory activity.

393. Using automated computer vision technology to study anticipatory activity in mouse

C. Hsu, K. Gunapala, D. Chang, A. Steele

Animals in nature show an extraordinary ability to sense time and predict future events. We are using the mouse as a model system to understand the neural basis of anticipatory behaviors. We have worked with several different models for anticipation: 1) anticipation of daily food during chronic calorie restriction; 2) anticipation of water access on chronic temporally restricted water access and 3) anticipation of social/sexual activity during daily timed female access for male mice. We measure anticipatory activity using a commercial computer vision system and are able to automatically describe the fine details of home cage behavior, including, food bin entry, drinking, hanging, jumping, walking, and grooming. Recently, we have been collaborating with computer vision scientists (T. Serre, H. Jhuang, E. Garrote, and T. Poggio) to develop an open source computer vision system to describe the fine details of home cage behaviors in mice automatically. Preliminary results of this system and its application to the study of anticipatory activity were presented.

Presented at Society for Research on Biological Rhythms, May 2010

394. Multisensory integration in the medicinal leech

D. Wagenaar

Extracting and evaluating environmental cues to guide behavioral decisions is a critical function of all nervous systems. Most animals combine cues from multiple senses to optimize their choices. For instance, an owl might combine visual and auditory cues to locate a mouse. Depending on external circumstances (light levels, wind), the two modalities may be weighted differently. Mammals and other vertebrates have evolved sophisticated brain structures to perform the requisite computations, but this very sophistication renders them less suitable for studying fundamental aspects of how nervous systems perform multisensory integration. Fortunately, animals with less complex nervous systems face similar challenges, and also must combine senses. The medicinal leech, a predatory worm, can use both visual and mechanical cues from water waves to locate its (mammalian) prey, and is ideal for studying multisensory integration because of the relative simplicity and accessibility of its nervous system. Leeches have dedicated mechanosensors for detecting water waves and can localize prey in complete darkness. They also have light sensors with which they can localize prey in the absence of mechanical cues. My lab studies the central neural circuitry to which these two sensory systems connect. Using quantitative behavioral analysis (work by Cynthia Harley) and a novel combination of multielectrode electrophysiology (electrode arrays fabricated by John Nagarah) and voltage-sensitive dye imaging, we investigate which aspects of the visual environment leeches respond to, how they weigh visual vs. mechanical cues, and how they determine the source of waves based on these cues.

Thanks to the simplicity of the leech's nervous system, it will be possible to map out the entire circuitry for multisensory integration—from sensory cells all the way to motor output—at the level of individual neurons and their connections. This will reveal key principles of how nervous systems in general can perform the calculations necessary for successful multisensory integration, and will suggest a framework for future experiments to study this important phenomenon in higher animals.

395. Synaptic mechanisms underlying hearing-related behaviors

G. Wu

Auditory System is one of the most important modules in our nervous system for performing daily tasks. We rely on this system to locate people, communicate with others, avoid potential dangers, and aesthetically, provide entertainment and pleasure by musical perception. My interests are focused on how auditory information is processed in our brain, and how the innate or acquired hearing deficits influence our hearing behaviors. My goal is to develop behavioral assays and conduct novel electrophysiological and imaging techniques for addressing those two questions. Over the past year, I worked with Dr. Li I. Zhang and his colleagues of USC to finish three studies. We investigated synaptic mechanisms underlying auditory cortical processing of rat. At the same time, I initiated two projects to link synaptic mechanisms to those specific hearing-related behaviors. Collaborating with Prof. Mark Konishi, I started my journey to neuroethology. Barn owl has been extensively studied because of their capability of using binaural cues to locate their prey in the dark. Auditory pathways involving nucleus magnocellularis (NM) and nucleus angularis (AN) in barn owl's brain stem are specialized to process timing and intensity information respectively. There exists one fundamental question: Why do neurons in those two nuclei show different response properties although each same auditory nerve bifurcates into two and innervates NM and AN neurons? This study will provide a unique opportunity to understanding the synaptic circuits for neurons in the earliest stage of central auditory processing that contribute to basic behaviors like sound localization. The other research line was also launched independently last year. It involves the characterization of ultrasound-evoked behaviors in mice, which is the first step to uncover how neural circuits process complex sound for vocalization and generate specific behaviors.

396. Inhibitory scale defines two complementary mechanisms for sound duration coding

G.K. Wu, M. Zhou[*], H. Tao[*], L. Zhang[*]

The coding of sound duration by neurons in the central auditory system can be simply achieved in two different ways: the neuron can respond continuously during the presentation of the sound stimulus (sustained response); or it can respond transiently to the onset and offset of the sound stimulus (phasic response). In the dorsal cochlear nucleus (DCN), using loose-patch cell-attached recordings, we found that principal neurons exhibited either sustained or phasic spike responses to tones of varies durations, similar as type III or type IV neurons described previously. *In vivo* whole-cell current-clamp recordings revealed that for "sustained-type" neurons, tone stimuli evoked sustained depolarization during the course of the stimulus, while sustained hyperpolarization was generated in "phasic-type" neurons with depolarization or spiking appeared only around the onset and offset of the stimulus. With whole-cell voltage-clamp recordings, we further dissected the excitatory and inhibitory synaptic inputs underlying the two types of tone-evoked responses. Both the "sustained-type" and "phasic-type" neurons exhibited long lasting excitatory and inhibitory synaptic currents during the course of the tone stimulation. The sustained depolarization/spiking responses are determined by the synaptic integration between strong excitatory and relatively weaker inhibitory inputs, while sustained hyperpolarization can be attributed to relatively stronger inhibition. Thus, the relative amplitudes between excitation and inhibition largely determine how the neurons respond to long-duration tone stimuli.

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397. Balanced excitation and inhibition in developing auditory

Y. Sun[*]*,G.K. Wu, H. Tao*[*]*, L. Zhang*[*]

An approximate balance between co-activated excitatory and inhibitory inputs is often observed for layer 4 neurons in adult sensory cortices, which has been proposed to be important for the information processing. However, little is known about how this important feature of neural circuits arises during development. In this study, *in vivo* whole-cell voltage-clamp recordings were systematically applied to layer 4 neurons of the primary auditory cortex at postnatal ages from P12 to P30. This covers the period from the opening of the ear canal when the sound evoked responses can be first recorded in the cortex, to the stage with the formation of adult like tonal receptive field. With tone stimuli at different frequencies and intensities, and by clamping the cells at 0 mV and -70 mV, respectively, we obtained the excitatory and inhibitory synaptic inputs evoked by the same tone stimulus. Consistent with the previous studies, the tone-evoked excitatory and inhibitory inputs exhibit high intensity threshold and long onset latencies. Both intensity threshold and onset latencies reduce progressively with the development. To our surprise, our data demonstrate that even at the earliest developmental stage (i.e., P12) examined, both excitatory and inhibitory inputs exhibit similar tonal receptive fields and frequency tuning, with inhibitory inputs temporally delayed from the excitatory inputs. The amplitudes of excitatory and inhibitory inputs covary with each other for the different tone stimuli. These observations suggest that the basic synaptic circuitry in the layer 4 of the auditory cortex is already formed even before the arrival of auditory inputs in the cortex. The auditory experience may still play a role in a later stage for the modification of a previously formed circuit to adapt to the specific acoustic environments.

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Thomas Hunt Morgan Senior Research Fellow: Katalin Fejes Toth
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Summary: The sequencing of eukaryotic genomes and transcriptomes revealed that a remarkably small fraction of both is occupied by protein-coding sequences (<2% in human). Instead, much of what was thought to be "junk DNA" turns out to encode for so called non-coding RNAs (ncRNA) that, similarly to proteins, regulate important biological processes. We use cell culture and fruit fly as models and a combination of biochemistry, molecular biology and high-throughput sequencing techniques to address how different classes of small non-coding RNAs regulate chromatin structure and transcription. Currently we are focusing on the role of two independent classes of small RNAs:

398. Biogenesis and function of genic small RNAs

We have recently described and characterized a new class of small RNAs in human cell lines, termed genic small RNAs, that map to the promoter regions (promoter associated small RNAs or PASRs) and the bodies of protein coding genes. Our studies revealed that the majority of protein-coding genes give rise to such short non-coding RNAs and expression levels of genic small RNAs, in general, correlates with the transcription of the corresponding mRNA and the presence of an active chromatin state. While the function of genic small RNAs is still unclear, several lines of evidence indicate that the RNAs or the act of their transcription *per se* regulate the expression of the protein-coding genes to which they are linked.

We wish to investigate how the expression of these newly identified non-coding RNAs is controlled and how they in turn affect expression of protein-coding genes. We are currently developing new tools to **1)** identify pathways involved in their biogenesis and elimination and **2)** identify their functional role.

399. Piwi-mediated epigenetic regulation in the *Drosophila* germline

Establishing the correct chromatin state is crucial for maintaining the genomic integrity of the germline. Piwi proteins and their small RNA partners, the Piwi interacting RNAs or piRNAs, function in the germline to repress transposon activity thereby maintaining genomic integrity. Much is known about the cytoplasmic function of Piwi proteins where they repress expression of transposable elements by cleavage of transposon mRNA. All animals express at least one member of the Piwi protein family in the nucleus, but the biological significance of this localization is not known. We are testing the hypothesis that Piwi exerts its effects on transposon expression in part by altering the epigenetic state and transcription of transposable elements. We are using high-throughput sequencing methods to identify genomic loci at which Piwi is enriched and are testing the effect of Piwi depletion on histone modification patterns.

Facilities

Flow Cytometry and Cell Sorting Facility
Genetically Engineered 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/Peptide Microanalytical Laboratory

Flow Cytometry and Cell Sorting Facility
Facility Manager: Rochelle Diamond
Faculty Supervisor: Ellen V. Rothenberg
Sorting Operator: Diana Perez
Optics and Maintenance Specialist: Patrick Koen

400. The Caltech Flow Cytometry/Cell Sorting Facility is located in Kerckhoff 020 and 026. The mission of the facility is to foster scientific research by providing the expertise, state-of-the-art resources, and training necessary to solve complex biological research problems and promote cutting edge research on a fee-for-service basis. The facility strives to provide cost effective analysis and cell separation on several different platforms using a myriad of protocols to enhance the scope and quality of the investigator's research.

The facility is equipped with two research grade flow cytometer cell sorters and one analyzer. This instrumentation can analyze and separate various types of cells and micro-organisms according to their measurable properties of light scatter and fluorescence. The BD FACSAria IIu is capable of analyzing at least nine colors utilizing three lasers (407nm, 488nm, and 633nm), and of carrying out 4-way sorting up to 10,000 cells per second with reliable efficiency and recovery, or 1-way sorting, such as for single-cell cloning, into various cell culture plate configurations. The iCyt Mission Technology Reflection 5-laser/9color (UV, 405, 488, 561, and 633nm) cell sorter with one Highly Automated Parallel Sorting (HAPS) module is contained in a Baker Sterilguard Advance Biosafety cabinet (BSL2). The BD FACSCalibur is 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 provides consultation services to all researchers on issues relating to flow cytometry, cell sorting, and cell separation techniques (173 consultation appointments with 32 Caltech lab groups and 17 administrative, JPL, and external consultations). In addition, the facility makes Treestar's FlowJo off-line analysis program available to its clients (39) and non-clients (15) through a network license. The facility has negotiated discounts with two antibody vendors and placed 54 orders for its clients this past year.

This past two years the facility provided service to 31 laboratories from the Divisions of Biology, Chemistry and Chemical Engineering, Applied Physics, Geology and Planetary Science, and JPL. In all, the projects of 81 individual users were supported. Twenty two researchers were trained in flow cytometry and the use of the BD FACSCalibur analyzer.

Research applications

In the past year, the Facility has been used by multiple groups for diverse applications. This is a representative sample of the projects under way. Tobias Heinen, Jongmin Nam, Dave McClay and Eric Davidson have been working with the facility to gain a new, improved sample preparation of sea urchin embryos for cell sorting after microinjection. This is a major effort to transform experimental technology for *cis*-regulatory analysis of these disaggregated sea urchin embryos bearing sets of >100 uncharacterized bar-coded cis-regulatory constructs and also expressing a fluorescent marker gene that identifies a given regulatory domain of the embryo. Those of the uncharacterized constructs that are expressing in the marked domain can be identified following disaggregation and isolation of the active cells by FACS.

Jingli Zhang of the Rothenberg Group has initiated collaboration with the Wold group to develop a comprehensive genome-wide map of the changes in epigenetic markings across the early stages of T-cell development. She has optimized conditions for *in vitro* expansion of early T-cell precursors at defined stages derived from fetal liver precursors. The facility then sorts ~10^7 cells of specific phenotypic subsets that will be used for downstream chromatin immune precipitation with a range of histone modification and transcription factor specific antibodies. These samples are then converted to Solexa sequencing libraries and sequenced by the staff of the Jacobs Genome Center, with valuable guidance from the Wold group and from Ali Mortazavi. The results to date are revealing a remarkably illuminating view of the genomic dynamics of a cell type specification process.

Akiko Kumagai and Zheng Meng of the Dunphy Lab has been using flow cytometry to examine siRNA treated cells to look at DNA replication by a new Click-iTsystem from Invitrogen for labeling S phase of the cell cycle. This new technique combined with DNA staining for cell cycle analysis has enabled the group to look at checkpoints in DNA replication and checkpoint regulation in mammalian and *Drosophila* cell systems.

The facility has been working closely with Arya Khosravi of the Mazmanian group to work out new types if sample preparations, staining protocols, magnetic bead enrichment for intestinal immune cells for analysis and cell sorting. Other members of the group are routinely using cell sorting for $CD4^+CD45Rb^{high}$ or $CD4^+CD45Rb^{low}$ T cells for injection into recipient mice to assess whether mice treated with a unique carbohydrate molecule Polysaccharide A from b.fragilis are protected from inflammatory bowel disease and colitis. Mazmanian lab members also make extensive use of the self-service analyzer provided by the facility to assay for Tregs, FoxP3, and pro-inflammatory cytokine production.

David Sprinzak, Lauren Lebon, and Leah Santat of Elowitz group are studying how the signaling pathways and gene regulatory dynamics work together. They are designing new developmental circuit designs using the signals for Notch and Delta regulation during development employing fluorescent reporters as a read-out for Notch activation by a plate-bound Delta protein. They use the cell sorting facility to sort these newly designed single cells to create stable cell lines of CHO and MDCK cells expressing the fluorescent reporters, mCherry, CFP, and YFP. In addition Fred Tan and John Yong are exploring

the regulatory pathways that maintain mouse embryonic stem cells in their pluripotent state. They perturb the pathway and use the sorter to purify cells and analyze their molecular transitions.

Mary Yui of the Rothenberg Group utilizes single cell deposition sorting in conjunction with the multiple fluorescence capabilities of the FACSAria IIu sorter to purify numerically rare early T cell populations for two main purposes: (1) to study the developmental potential and lineage choices of these precursor cells in a cell co-culture system, and (2) to determine gene expression patterns of these specific early T cell populations in the context of gene regulatory networks in T cell development in a mouse model of Type 1 diabetes.

Jonathan Moore of the Rothenberg group has been working with the facility to enrich lamprey cells expressing the variable lymphocyte receptor, as well as negative control cells. These cells are used to create nuclear extracts for gel shift experiments, RNA for qRT-PCR analysis, and DNA-protein complexes for chromatin immunoprecipitation experiments.

Long Li and other Rothenberg lab members have been studying the effects of reducing the activity of particular transcription factor genes at defined stages of T-cell development *in vitro*. To do this, they initiate differentiation cultures with progenitor cells sorted from day 13.5 murine fetal liver, and then at particular time points introduce shRNA by transfection or retrovirally infect the cells with Cre, to excise a floxed transcription factor gene. Cells are then resorted to purify transfected cells and returned to culture for defined periods, then re-sorted for purifying the resulting developmental subsets from normal and control cells. Based on 4-6 color staining cells are characterized for their alteration in RNA expression and further developmental potential. The facility must book these appointments up to a month ahead of time for this kind of time-dependent sorting and analyses.

The Heath lab relies heavily on the facility for flow cytometry and sorting to provide validation for a host of new approaches and biological methods that are currently under development. The areas under investigation include: (1) Developing intracellular signaling network hypotheses to help understand the tumor microenvironment; and (2) Strategies for the multiplexed sorting of CD8+ antigen specific T-cells for applications that include cancer immunotherapy applications. Most of their developed methods are chemical approaches that are reduced into microfluidics environments. FACS is one of the primary assay techniques for validating these approaches.

Elaine Hsaio and Mimi Sadoshima in the Patterson group have been working hard with the support of the facility to find enrich for very rare placenta stem cells prior to sorting for $CD34^{+}$/c-kit^{+} hematopoetic stem cells (HSCs). They are examining placenta and fetal livers of control and maternal inflammatory activated (MIA) offspring using markers associated with particular hematopoietic multipotent progenitors to look at differential lineage priming of HSCs. They will test *in vitro* effects of poly(I:C) and inflammatory cytokines on these sorted HSCs. By evaluating the effects of MIA on embryonic HSC self-renewal, differentiation, homing, and premature exhaustion of the HSC pool, they hope to gain insights into the long-term implications of MIA on immune dysregulation in schizophrenia and autism.

The facility supported the R&D of Young-Kyung Bae and Snehalata Kadam of the Stathopoulos group who are using the new iCyt cell sorter to isolate various types of migrating cells from transgenic *Drosophila* embryos for RNA expression, to identify signaling pathways that influence development. This is a challenge because dissociated *Drosophila* embryo cells expressing transgenic markers of interest are very rare compared to the non-expressing cells and debris.

Flow Cytometry/Cell Sorting Facility publications

Chow, J., Mazmanian, S.K. (2010) A pathobiont of the microbiota balances host colonization and intestinal inflammation. *Cell Host Microbe.* **7**(4):265-276.

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Yun Kyung Leea, Juscilene S. Menezesa, Yoshinori Umesakib, and Mazmanian, S.K. (2010) Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Published online before print July 26, 2010, doi: 10.1073/*PNAS.*

Genetically Engineered Mouse Production Facility
Director; Member of the Professional Staff:
Shirley Pease
Mouse Colony Mgr., Cryopreservation, Rederivation: Jennifer Alex
Microinjection: John Earle
Embryonic Stem Cell Culture: Simon Webster

401. 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 the 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 in 2007 to the pioneers of this technology, Mario Capecchi, Martin Evans and Oliver Smithies.) The facility, in collaboration with Anderson, Baltimore, Fraser, Kennedy, Lester, Patterson, Rothenberg, Simon, Varshavsky and Wold laboratories, has generated multiple transgenic, knockout and knockin mouse strains, amounting to nearly 180 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.

With regard to the injection of DNA into pro-nuclei of pre-implantation stage embryos, this year, GEMs staff have assisted the Fraser lab in an early embryonic developmental study of Oct 4 kinetics, for the prediction of cell lineage patterning, by the injection of DNA into single nuclei of embryos at 2-cell stage, or into the cytoplasm of 2-cell stage blastomeres. The work has been published online: Kinetics predict cell lineage patterning in the early mammalian embryo. *Nature Cell Biology*, October 4, 2011, doi10.1038/ncb2154.

In tissue culture and the use of embryonic stem (ES) cells the Facility has 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 were derived for the Rothenberg lab. The Facility is able to offer investigators a choice between working with human or murine ES cells. Human ES cell lines from WiCell, H1 and H9, are available. We have multiple murine ES cell lines available for use. Several are on a 129 background, some on a C57BL/6 background and others are F1 cell lines, which are a mix between 129 and C57BL/6 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 2-year breeding program to reach the same point, having initially established the mutation on a sub-optimal genetic background. However, in general, a lower proportion of targeted clones from C57 ES cell lines can be expected to go germline. 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. This year, we assisted the Rothenberg lab by use of this technology. This is made possible by first, the production of tetraploid embryos. These are made by fusion of two blastomeres at the 2-cell embryo stage, resulting in the production of a single viable blastomere that has twice the normal number of chromosomes. Such embryos can develop to blastocyst stage, but thereafter, can only contribute to extraembryonic cell lineages. Thus, ES cells injected into the blastocoel cavity in this case, are sole contributors to the developing embryo. Not every ES cell line is able to support development to such a degree. 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.

In the light of recent publications, we are currently evaluating the benefit of altered ES cell culture conditions, by supplementing media with insulin and two kinase inhibitors. Our goal is to make our gene targeting system more robust and efficient. Also in tissue culture this year we derived and immortalized a fibroblast cell line

from a genetically altered mouse strain for the Baltimore lab. We also organized, set up and taught a 3-week course for ten "Bridges to Stem Cells" students. This was in conjunction with PCC and funded by CIRM. Students had the opportunity to derive fibroblasts and ES cell lines, plus execute a gene targeting experiment. The students successfully developed a GFP expressing ES cell line that will be useful for teaching at PCC in the Biotechnology course, which is directed by Pam Eversole Cire, (former Caltech postdoctoral scholar).

Once a new mouse model has been characterized, it may be cryopreserved by GEMs staff, or sent to the Mutant Mouse Resource Center, to be made available to the research community in general. We currently have over 100 mouse models cryopreserved. For each line, between 200 and 500 embryos at 8-cell stage have been preserved in liquid nitrogen. There are currently 34,752 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. We also offer re-derivation as a service, whereby investigators can bring in novel mouse strains from other Institutions without risk of introducing pathogens to CIT stocks. This involves the washing and transfer of pre-implantation embryos from "dirty" incoming mice to "clean" CIT recipient animals.

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. GEMs Facility staff have been working with IMSS in the development of 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 and most valuable for the reports the system will be able to generate. We anticipate this will be a very useful animal management tool and anticipate launching the software early next year.

Following, is a list of the names of the seventeen principal investigators and their postdoctoral fellows or graduate students who are presently using GEMs services.

David Anderson
Haijiang Cai, Ben Deneen, Wulf Haubensak, Prabhat Kunwar, Hyosang Lee, Dayu Lin, Li Ching Lo, Agnes Lukaszewic, Sophia Vrontu

Alexei Aravin
Dubravka Pezic

David Baltimore
Alex Balazs, Mark Boldin, Aadel Chaudhuri, Shengli Hao, Eun Mi Hur, Daniel Kahn, Jocelyn Kim, Xin Luo, Ryan O'Connell, Param Ramakrishnan, Alex So, Lili Yang, Jimmy Zhao

Mark Davis - (Chemistry and Chemical Engineering)
Jonothan Choi, Han Han, Leonard Medrano, Jonothan Zuckerman

Ray Deshaies
Narimon Harnapour

Michael Elowitz
Julia Tischler

Scott Fraser
Max Ezin, David Koos, Periklis Pantazis, Nicholas Plachta, Carol Readhead

Linda Hseih Wilson
Joshua Brown, Peter Clarck, Chithra Krishnamurthy, Claude Rogers, Andrew Wang, BinQuan Zhuang

Mary Kennedy
Holly Carlisle, Tinh Luong, Edoardo Marcora, Leslie Schenker

Cristof Koch
Andrew Steele

Henry Lester
Purnima Deshpande, Ryan Drennan, Herwig Just, Elisha Mackay, Sheri McKinney, Julie Miwa, Rachel Penton

Sarkis Mazmanian
Sarah McBride

Paul Patterson
Ben Deverman, Elaine Hsiao, Jan Ko, Ali Koshnan, Natalia Malkova, Limin Shi, Sohila Zadran

Ellen Rothenberg
Long Li, HaoYuan Kueh, Mary Yui, Jingli Zhang

Melvin Simon
Sang-Kyou Han

Alexander Varshavsky
Christopher Brower, Konstantin Platkov, Brandon Wadas

Barbara Wold
Brian Williams

Publication

Plachta, *et al.* (2011) Oct4 kinetics predict cell lineage patterning in the early mammalian embryo. *Nature Cell Biology*, doi10.1038/ncb2154.

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Millard and Muriel Jacobs Genetics and Genomics Laboratory
Director: Igor Antoshechkin
Staff: Brandon King, Vijaya Kumar, Lorian Schaeffer

Support: The work described in the following research at the Laboratory has been supported by:
Millard and Muriel Jacobs Family Foundation

402. The Millard and Muriel Jacobs Genetics and Genomics Laboratory provides support for genomics research to the Caltech community with an emphasis on high throughput sequencing and microarray analysis. During the period of this report, the Laboratory has worked with groups from the Division of Biology, the Division of Chemistry and Chemical Engineering and the Division of Engineering and Applied Sciences.

Research Support: *Division of Biology* - The Laboratory performed high throughput sequencing experiments for the groups of professors Angela Stathopoulos, Barbara Wold, Bruce Hay, David Chan, Ellen Rothenberg, Elliot Meyerowitz, Eric Davidson, Grant Jensen, John Allman, Jose Luis Riechmann, Mary B. Kennedy, Michael Elowitz, Paul Patterson and Paul Sternberg. The projects ranged from the analysis of mtDNA mutation spectrum in skeletal muscle in mice (David Chan) to the *de novo* sequencing of nematode genomes (Paul Sternberg) and transcriptome analysis during flower development in *Arabidopsis* (Elliot Meyerowitz). Microarray experiments were carried out for the laboratories of David Baltimore and Sarkis Mazmanian.

Division of Chemistry and Chemical Engineering - The Laboratory manufactured carbohydrate microarrays and performed Affymetrix gene expression profiling for the Hsieh-Wilson group. ChIP-Seq experiments and mutation screens using Illumina HTS platform were performed for the laboratories of Peter Dervan and Judith L. Campbell, respectively.

Division of Engineering and Applied Science - The Laboratory conducted HTS experiments for the laboratory of Jared R. Leadbetter, including genome sequencing and transcriptome analysis of multiple *Treponema* strains.

Infrastructure and capabilities: The Laboratory operates two Illumina GAIIx high throughput sequencers that allow us to perform a wide variety of experiments, including ChIP-Seq, RNA-Seq, small RNA analysis, *de novo* genome sequencing, mutation discovery, etc. The Laboratory has all the necessary equipment to support the HTS workflow, including analytical instruments such as Agilent 2100 Bioanalyzer, LightCycler 480 qPCR system, Qubit fluorometer and Nanodrop ND-1000 spectrophotometer that are used for the sample quality assessment and library validation.
Some of the equipment involved in microarray work includes Affymetrix GeneChip system, Agilent and GenePix Microarray Scanners, multiple hybridization ovens, etc. The Laboratory has the ability to manufacture custom microarrays using MicroGrid II arrayer, which have been recently used for studies of carbohydrate – protein interactions.

The Laboratory has developed an extensive computational infrastructure that allows us to carry out sequence data extraction using the Illumina Sequence Analysis Pipeline and to perform such computation-intensive secondary analyses as identification of binding sites for DNA-interacting proteins, genome assembly, transcriptome analysis, etc. A local copy of UCSC Genome Browser allows us to visualize HTS data within the context of genomic annotations. Resolver software suite (Rosetta Biosoftware) is available to our users for the analysis of microarray-based gene expression data. Additional microarray and HTS software tools and analysis packages, both public and commercial, are also available.

Publications acknowledging the laboratory:

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Monoclonal Antibody Facility
Supervisor: Paul H. Patterson
Director: Susan Ker-hwa Ou
Staff: Shi-Ying Kou

403. 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 while using small amounts of antigen. In addition to these services, 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. Transmembrane Bioscience obtained mAbs and polyclonal ascites against recombinant proteins from Bartonella, Scrub Typhus, *Orientia tsutsugamushi*, Coxiella Burnetti, Coxiella AdaA, and ESAT 6 (antigen from Mycobacterium tuberculosis). The Zinn laboratory obtained polyclonal ascites against *Drosophila* Toll 7.

As part of the NHGRI ENCODE project with Dr. Wold's group, we aim to produce more than 50 chromatin immunoprecitation (ChIP) validated mAbs against transcription factors for which ChIP-grade reagents are not available. To increase the success rate of usable reagents, we tested antigens fixed by formaldehyde in the same manner as is used in ChIP studies. Animals immunized with fixed and unfixed NRSF N250 were fused and mAbs were obtained from both groups. Therefore, we immunize animals with fixed Ag only. After cleaving the GST from the fusion protein, there is always some GST left in the sample, and several mice generated responses against the GST instead of the antigen. The adult mouse tolerization method (Lebron *et al.*, 1999) was applied with the MYF5 antigen and mice showed significant improvement in recognizing the Ag. Therefore, we tolerize mice with GST before immunization. Using these procedures, we obtained mAbs against NRSF N250, N150, C250, FOX P2 Ag1, FOX P2 Ag2, and GABPA 200-310 inclusion body, HES1, PER1, MYF5, BHLH, and FOXM1.

We are also currently working with the following groups: The Wold laboratory is immunizing with the human transcription factors NANOG, RBPJ, RORC, STAT, CSDA, NCOR, and RORC. Transmembrane Bioscience is immunizing with recombinant proteins from Rickettsia typhi OmpB and Crimean-Congo hemorrhagic fever (CCHF). The Chan laboratory is immunizing with human TFAM (mitochondria transcription factor A). The Jung laboratory from USC is immunizing with Rubicon.

Publications

Gardberg, A.S., Dice, L.T., Pridgen, K., Ko, J., Patterson, P.H., Ou, S., Wetzel, R. and Dealwis, C. (2009) Structures of Aβ-related peptide-monoclonal antibody complexes. *Biochem.* **48**:5210-7.

Publications utilizing work prepared by the Facility:

Bugga, L., Ratnaparkhi, A. and Zinn, K. (2009) The cell surface receptor Tartan is a potential *in vivo* substrate for the Receptor Tyrosine Phosphatase Ptp52F. *Molec. Cell Biol.* **29**:3390-400.

Hattori, J., Chen, Y., Matthews, B.J., Salwinski, L., Sabatti, C., Grueber, W.B. and Zipursky, S.L. (2010) Robust discrimination between self and non-self neurites requires thousands of Dscam1 isoforms. *Nature* **461**:644-8.

Legleiter, J., Lotz, G.P., Miller, J., Ko, J., Ng, C., Williams, G.L., Finkbeiner, S., Patterson, P.H. and Muchowski, P.J. (2009) Monoclonal antibodies recognize distinct conformational epitopes formed by polyglutamine in a mutant huntingtin fragment. *J. Biol. Chem.* **284**:21647-58.

Menon, K.P., Andrews, S., Murthy, M., Gavis, E.R. and Zinn, K (2009) The translational repressors nanos and pumilio have divergent effects on presynaptic terminal growth and postsynaptic glutamate receptor subunit composition. *J. Neurosci.* **29**:5558-72.

Ralston, K.S., Kabututu, Z.P., Melehani, J.H., Oberholzer, M. and Hill, K.L. (2009) The *Trypanosoma brucei* flagellum: moving parasites in new directions. *Ann. Rev. Microbiol.* **63**:335-62.

Southwell, A.L., Ko, J. and Patterson, P.H. (2009) Intrabody gene therapy ameliorates motor, cognitive, and neuropathological symptoms in multiple mouse models of Huntington's disease. *J. Neurosci.* **29**:13589-602.

Reference

Lebron J., Shen H., Bjorkman P. and Ou, S. (1999) *J. Immunol. Meth.* **222**:59-63.

Nucleic Acid and Protein Sequence Analysis Computing Facility, Biology Division, Caltech
Manager: David R. Mathog, Manager
Supervisor: Stephen L. Mayo

404. 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 EMBOSS-Explorer 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, PyMol, Molscript, 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. A web interface allows common compute intensive jobs to run 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, and, ChemSketch.

Protein Expression Center
Director: Jost G. Vielmetter
Supervisor: Barbara J. Wold
Faculty Advisors: Pamela J. Bjorkman, Mary B. Kennedy
Staff: Michael Anaya, Timothy Feliciano, Clarke Gasper, Inderjit Nangiana

405. The Protein Expression Center (PEC) was established in 1996 to provide protein expression and purification for Caltech and outside researchers. The center provides heterologous expression of recombinant proteins using *E. coli*, insect cells (Baculovirus) and mammalian cells (HEK 293).

The PEC has evolved over the last four years to provide additional capabilities that include expression optimization using multiwell-plate based miniaturization and parallelization. We also enhanced our purification and analytical capabilities and more recently we assist in developing and applying automated plate based biochemical protein and cell based bioassays. We continue to provide support in the experimental design and execution for Surface Plasmon Resonance (SPR) based measurements of protein-protein interactions or generally of bio-molecular interaction studies using a Biacore T100 instrument. The interest and use of this instrument has steadily increased and has become a valued asset in the Caltech research community.

The majority of proteins produced in the mammalian expression system are active human antiviral (influenza and HIV) antibodies and engineered antibody derivatives and viral glycoproteins (DARPA, Steven Mayo and Engineering Immunity, Pamela Bjorkman). Mainly we use protein expression based on transient DNA transfection but recently we succeeded in generating stable cell lines expressing anti-HIV antibodies and in one case a tRNA-synthetase mutant allowing incorporation of non-natural amino acids into expressed proteins in these cell lines.

We produced many "CHIP-able" mAbs for the ENCODE project, (Barbara Wold). "CHIP-able" mAbs are monoclonal antibodies capable of genome wide extraction and characterization of transcription factor specific DNA control sites. We have developed a production pipeline to generate antibodies in mice and llamas that are then screened for transcription factor specificity using robotic liquid handling technology. As a proof of concept we have so far been able to generate "CHIP-able" mAbs against the human transcription factor NRSF. We have produced a total of over a hundred monoclonal antibodies against transcription factors BHLHB2, CSDA, FOX-M1, FOX-P2, GAPBA, HES1, MYF5, NANOG, NRSF, PER1, RBPJ. We are currently focusing on the characterization of the CHIP-ability and other properties of those mAbs.

Publications that acknowledge the PEC in 2009/2010:

Pamela J. Bjorkman group (mammalian cell expression, baculovirus expression and Biacore support):

Diskin, R., Marcovecchio, P.M. and Bjorkman, P.J. (2010) Structure of a clade C HIV gp120 plus CD4 and a CD4-induced antibody reveals anti-CD4 polyreactivity. *Nature Struct. Mol. Biol.* **17**:608-613. doi:10.1038/nsmb.1796.

He, Y., Jensen, G.J. and Bjorkman, P.J. (2009) Cryoelectron tomography of homophilic adhesion mediated by the neural cell adhesion molecule L1. *Structure* **17**:460-471, doi:10.1016/j.str.2009.01.009.

Klein, J.S., Gnanapragasm, P.N.P., Galimidi, R.P., West, A.P., Jr. and Bjorkman, P.J. (2009) Examination of the contributions of size and avidity to the neutralization mechanisms of the anti-HIV antibodies b12 and 4E10. *Proc. Natl. Acad. Sci. USA* **106**:7385-7390. doi:10.1073/pnas.0811427106.

Klein, J.S., Webster, A., Gnanapragasam, P.N.P., Galimidi, R.P. and Bjorkman, P.J. (2010) A dimeric form of the HIV-1 antibody 2G12 elicits potent antibody-dependent cellular cytotoxicity. *AIDS* **24**:1633-1640. doi:10.1097/QAD.0b013e32833ad8c8.

West, A.P., Jr., Galimidi, R.P., Foglesong, C.P., Gnanapragasam, P.N.P., Klein, J.S. and Bjorkman, P.J. (2010) Evaluation of alternative CD4-CD4i antibody architectures yields potent, broadly cross-reactive anti-HIV reagents. *J. Virol.* **84**:261-269. doi:10.1128/JVI.01528-09.

West, A.P. Jr., Galimidi, R.P., Foglesong, C.P., Gnanapragasam, P.N.P., Klein, J.S., Suzuki, M., Tiangco, N.E., Vielmetter, J. and Bjorkman, P.J. (2009) Design and expression of a dimeric form of the anti-HIV antibody 2G12 with increased neutralization potency. *J. Virol.* **83**:98-104.

Yang, Z., West, A.P. Jr. and Bjorkman, P.J. (2009) Crystal structure of TNFalpha complexed with a poxvirus MHC-related TNF binding protein. *Nature Struct. Mol. Biol.* **16**:1189-1191. doi:10.1038/nsmb.1683.

Ray Deshaies group (baculovirus expression):

Kleiger, G., Hao, B., Mohl, D.A. and Deshaies, R.J. (2009) The acidic tail of the CDC34 ubiquitin-conjugating enzyme functions in both binding to and catalysis with ubiquitin ligase SCFCDC4. *J. Biol. Chem.* **284**:36012-36023.

Kleiger, G., Saha, A., Lewis, S., Kuhlman, B. and Deshaies, R.J. (2009) Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates. *Cell* **139**:957-968.

Pierce, N., Kleiger, G., Shan, S. and Deshaies, R.J. (2009) Detection of sequential ubiquitination on a millisecond time-scale. *Nature* **462**:615-619.

***Douglas Rees group (baculovirus expression and Biacore support)*:**

Lewinson, O., Lee, A.T., Locher, K.P. and Rees, D.C. (2010) A distinct mechanism for the ABC transporter BtuCD-BtuF revealed by the dynamics of complex formation. *Nature Struct. Molec. Biol*. **17**:332-338. doi:10.1038/nsmb.1770.

Rice, A.E., Mendez, M.J., Hokanson, C.A., Rees, D.C. and Bjorkman, P.J. (2009) Investigation of the biophysical and cell biological properties of ferroportin, a multipass integral membrane protein iron exporter. *J. Mol. Biol*. **386**:717-732. doi:10.1016/j.jmb.2008.12.063.

Protein/Peptide MicroAnalytical Laboratory
Director: Jie Zhou
Faculty Advisor: Mary J. Kennedy
Associate Biologist: Felicia Rusnak

406. 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; and development of Os based column for the on-column digestion of protein.

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); and, MASCOT server

New Development: One recent accomplishment was the identification and quantitation of N-terminal acetylation of the protein from Alexander Varshavsky's group. Their paper, with input from our analysis work, was recently published: Hwang, C.S., Shemorry, A. and Varshavsky, A. (2010) N-terminal acetylation of cellular proteins creates specific degradation signals. *Science* **327**(5968):973-977.

We have been investigating insoluble and cross-linked $[Os(dmebpy)_2Cl]^{+/2+}$-derivatized copolymer of acrylamide and vinylimidazole, and found some novel functions with biological application. The resulting paper has been published. The paper's title and abstract is as following:

Abstract

Fast trypsin digestion of proteins on a cross-linked [Os(dmebpy)2Cl]+/2+-derivatized copolymer of acrylamide and vinylimidazole column.

Fast digestion of proteins was observed when they were loaded together with trypsin onto the cross-linked [Os(dmebpy)2Cl]+/2+-derivatized copolymer of acrylamide and vinylimidazole column. The insoluble Os-complexed polymer particles were packed into an electrospray tip to monitor peptides eluted during loading, washing and elution periods with a mass spectrometer. The proteolytic cleavage of proteins was observed immediately when the mixture of trypsin and substrates in 0.2 mM ammonium bicarbonate 50/50 H2O/ACN reached the column tip, and continued through the loading period. Some tryptic peptides were released from the column during the loading and following washing periods. The others still stayed on the column until the low pH elution buffer reached the column. If a protein was first loaded onto the column, no tryptic peptides of the protein were observed when trypsin was loaded later for the on-column digestion. Only the autolysis peptides of trypsin were observed. On-column digestion of 100 fmol myoglobin was successfully detected with a low sensitivity quadrupole mass spectrometer. A hybrid Os-polymer/ C18 column tip was constructed for the online trypsin digestion of proteins in the aqueous buffers and the following trapping and elution of peptides from C18 column. The digestion of reduced and alkylated bovine serum albumin and human transferrin in 2.5 mM ammonium bicarbonate and 0.2 M urea buffer was observed on the column, with more peptide coverage than conventional 4 hour in-solution digestion at 37ºC. Control experiments without Os-polymer in the column tip excluded the spontaneous in-solution digestion of proteins in the short time window of buffer delivery onto the column, indirectly confirming the contribution of Os-polymer on the fast trypsin digestion.

Services

During the first eight months of fiscal 2010 PPMAL interacted with 20 laboratories. Samples were analyzed from the Division of Biology, and Chemistry and Chemical Engineering (see list). A total of 712 samples were analyzed. In addition to our work for campus faculty and staff, work was also performed for off-campus institutions (43 samples analyzed).

PPMAL
October, 2009 - May, 2010

On-Campus

	No. of Samples	No. of Mass	No. of Proteomics	#Seq.	No. of Seq. Cycles
Baltimore	4		1	3	20
Barton	4	4			
Bjorkman	3			3	56
Chan, David	5	2		3	12
Clemons	16	6	9	1	15
Davis, Mark	1	1			
Dervan	3	3			
Deshaies	5			5	25
Elowitz	14	6	8		
Goddard	2	2			
Gray	170	166	2	2	39
Grubbs	1	1			
Heath	358	358			
Hsieh-Wilson	27	27			
Kennedy	1		1		
Mayo	20	20			
Patterson	6		6		
Rees	28	2	25	1	20
Tirrell	13	8	3	2	6
Varshavsky	31	2	29		
TOTALS	**712**	**608**	**84**	**20**	**193**

Off-Campus

Integrated Diagnostics: former James Heath, Harry Gray students	6	6			
Glyport: former Scott Fraser postdoc.	28	28			
Trans Membrane Biosciences: former Sunney Chan student	1		1		
Carleton University: former Jacqueline Barton postdoc.	1	1			
Princeton University-Prof. James Link: former David Tirrell postdoc.				3	
Epoch Biosciences	4	4			
TOTALS	**43**	**39**	**1**	**3**	

Graduates

DIVISION OF BIOLOGY

Doctor of Philosophy, 2010

Holly C. Beale, Ph.D.
Biology
B.A., Columbia College at Columbia University 1991.
Thesis: Synaptic Signal Transduction and Transcriptional Control.

Paola A. Betancur, Ph.D.
Biology
A.S., Suffolk Community College 2002; B.S., Stony Brook University 2003.
Thesis: *Cis*-regulatory Analysis of the Key Developmental Gene, SOX1O, in Neural Crest and Ear.

Gil Bastos de Carvalho, Ph.D.
Biology
M.D., University of Lisbon Medical School 2002.
Thesis: *Drosophila* Feeding Behavior and Demographic Mechanisms of Lifespan Extension.

Chiraj Kiran Dalal, Ph.D.
Biochemistry and Molecular Biophysics
B.A., University of Pennsylvania 2003.
Thesis: Causes and Consequences of Gene Expression Noise.

Daniel A. Gold, Ph.D.
Biology
B.A., Cornell University 2000.
Thesis: Molecular Characterization of the Dbf4/Drf1-Dependent Kinase (DDK) and the DNA Replication Checkpoint Mediator Claspin in *Xenopus* Egg Extracts.

Jennifer Jin Lee Hodas, Ph.D.
Biochemistry and Molecular Biophysics
B.A., B.S., Yale College 2004.
Thesis: Elucidating the Hippocampal Dopaminergic Subproteome with Novel Bioorthogonal Techniques.

Hiroshi Ito, Ph.D.
Biology
M.D., Kyoto University 2003.
Thesis: Neuromodulator-mediated Control of Spatial and Nonspatial Information Processing in the Hippocampus.

Tinh Nghi Luong, Ph.D.
Biology
B.A., University of California, Berkeley 1997.
Thesis: Signaling Proteins in the Post-Synaptic Density.

Amy Jeanette McMahon, Ph.D.
Biology
B.S., University of Washington 2005.
Thesis: Fibroblast Growth Factors Influence Collective Cell Behavior during Mesoderm Migration.

Maria Papadopoulou, Ph.D.
Biology
B.A., Smith College 2001.
Thesis: Gain Control and Sparse Representations in the Olfactory System of the Locust and Fly.

Juan S. Ramírez-Lugo, Ph.D.
Biology
B.S., University of Puerto Rico, Mayaguez 2001.
Thesis: The Activation of ATR in Response to Double-Stranded DNA Breaks.

Dinesh Subba Rao, Ph.D.
Biology
B.S., Case Western Reserve University 1995; M.D., 2000.
Thesis: Small RNAs Play Big Roles in Hematopoietic Development.

Alice Anne Pennoyer Robie, Ph.D.
Biology
B.S., Brandeis University 2002.
Thesis: Multimodal Sensory Control of Exploration by Walking *Drosophila melanogaster*.

Jasper Chen Simon, Ph.D.
Biology
B.A., California State University, Fullerton 1996.
Thesis: Behavioral Analysis of Exploration and Dispersal in *Drosophila*.

Karen Elizabeth Wawrousek, Ph.D.
Biochemistry and Molecular Biophysics
B.S., The College of Saint Rose 2001.
Thesis: Contributions of DNA2 and the Tim/Tipin Complex to Genomic Stability.

Ashley Palani Wright, Ph.D.
Biology
B.S., The University of Utah 1999.
Thesis: Genetic Analysis of Axon Guidance in *Drosophila melanogaster*.

Suzuko Yorozu, Ph.D.
Biology
B.S., Concordia University 2001; M.S., University of Toronto 2004.
Thesis: Distinct Sensory Representations of Wind and Near-Field Sound in the *Drosophila* Brain.

Mark Andrew Zarnegar, Ph.D.
Biology
B.A., B.S., University of California, San Diego 1998.
Thesis: Investigating the Transcriptional Mechanisms Controlling *Sfpi1*, a Critical Regulatory Node within Multiple Lineage Specifying Subcircuits of the Hematopoietic Gene Regulatory Network.

Master of Science - Biology

Sarah Knox Gillespie *(Biology)* B.A., Washington University St. Louis 2004.

Sindhuja Kadambi *(Biology)* B.A., Rutgers University 2005.

Bachelor of Science, 2010

Maria Belyi *Newton, Massachusetts* **Biology**

Daniel Chang *Walnut, California* **Biology**

ZeNan Li Chang* *Santa Monica, California* **Biology**

Lu Chen* *Rockville, Maryland* **Biology**

Evelyn S. Chou *New City, New York* **Biology**

Diana Remy Dou* *Rosemead, California* **Biology** and Business Economics and Management and English (Minor)

Andrew Michael Freddo* *Colts Neck, New Jersey* Chemistry and **Biology**

Annie Van Hong* *La Puente, California* **Biology**

Daniel Leighton *Livingston, New Jersey* **Biology**

Jennifer Si Li* *Edmond, Oklahoma* **Biology**

Jason Robert Lunn *Miami, Florida* **Biology**

Micah John Manary* *St. Louis, Missouri* **Biology** and History

Nicholas Rosa† *Tupelo, Mississippi* Chemistry and **Biology**

Esther Shyu* *Naperville, Illinois* **Biology**

Benjamin Steele* *Oak Park, California* **Biology** and History (Minor)

Elaine E. To* *North Hollywood, California* **Biology**

Leslie Mae Tong* *Ventura, California* **Biology**

Jonathan Michael Tsai* *Saratoga, California* **Biology**

Kevin Thomas Welch† *Austin, Texas* Bioengineering (Synthetic **Biology**) and English

Lisa Yee* *Los Angeles, California* **Biology**

Lisa D. Zang *Albany, California* **Biology** and Business Economics and Management

**Students whose names are followed by an asterisk are being graduated with honor in accordance with a vote of the faculty.*

†*Students whose names are followed by a dagger are close to completion and will receive diplomas at the end of the academic year in which all graduation requirements are met.*

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FINANCIAL SUPPORT

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Agouron Moore Foundations
Al Sherman Foundation
American Heart Association
Anna L. Rosen Professorship
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Arnold and Mabel Beckman Fellowship
Arnold and Mabel Beckman Foundation
ARRA Administrative Supplement, National Institute of Aging
ARRA Administrative Supplement, National Institute of Neurological Disorders and Stroke
ARRA National Institute of Mental Health
ARRA National Institute on Drug Abuse
Arrowhead Grubstake Fund
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Autism Speaks Foundation

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Baxter Senior Postdoctoral Fellowship
Beckman Institute
Bill and Melinda Gates Foundation
Binational Science Foundation
James G. Boswell Professor of Neuroscience
Bren Foundation
Broad Foundation
Broad JCTM
Burroughs Wellcome Fund

California Institute of Regenerative Medicine (CIRM)
Caltech Center for Biological Circuits Design
Caltech Innovation Initiative
Caltech-City of Hope Biomedical Initiative
Caltech-City of Hope Biomedical Research Initiative
Caltech/UCLA Joint Center for Translational Medicine
Camilla Chandler Frost Fellowship
Cancer Research Institute/Irvington Postdoctoral Fellowship
Carl Zeiss Jena
CDMRP Breast Cancer
Christopher Reeve Foundation
CIRM
CIRM Bridges to Stem Cell Research at Pasadena City College
Colvin Fund for Research Initiatives in Biomedical Science
Crohn's and Colitis Foundation of America

Damon Runyon Cancer Research Foundation
DARPA Young Faculty Award
Defense Advance Research Project Agency (DARPA)
Defense University Research Instrumentation Program
Della Martin Foundation
Department of Defense (National Security Science and Engineering Faculty Fellowship)
DNA Sequencer Patent Royalty Funds
DOE
Donald Bren Foundation

Edelman Family Discovery Fund
Edward Mallinckrodt Jr. Foundation
Eli and Edythe Broad Foundation
Ellison Medical Foundation
Ellison Medical Foundation
European Molecular Biology Organization Fellowship

Foundation for NIH Research
G. Louis Fletcher
Mrs. Laura Frlan

Louis A. Garfinkle Memorial Laboratory Fund
Gimbel Discovery Fund
Gimbel Discovery Fund in Neuroscience
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Gordon and Betty Moore Foundation
Gordon Ross Fellowship
Gosney Postdoctoral Fellowship

Hanson Fund
Helen Hay Whitney Foundation
Hertz Fellowship
Hicks Alzheimer's Fund
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Howard Hughes Medical Research Institute
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G. Harold & Leila Y. Mathers Charitable Foundation
McGrath Foundation
McKnight Foundation
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Mettler Foundation
Ministry of Education
Multi University Research Initiative
Multiple Myeloma Research Foundation

National Aeronautics and Space Administration
National Eye Institute
National Heart, Lung and Blood Institute
National Human Genome Research Institute
National Institute for Biomedical Imaging and Bioengineering
National Institute of Child Health and Human Development
National Institute of General Medical Sciences
National Institute of Mental Health
National Institute of Neurological Disease and Stroke
National Institute on Aging
National Institute on Drug Abuse
National Institutes of Health (NIH)
National Institutes of Health (NINDS, NIDCR, NHGRI)
National Institutes of Health, USPHS
National Institutes of Mental Health
National Science Foundation (NSF)
NIH Director's Pioneer Award
NIH Program Project
NIMH
Norman Chandler Professorship in Cell Biology
NSF

Office of Naval Research

Packard Foundation
Peter Cross Gift
Pritzker Neurogenesis Research Consortium
Rosen Scholarships in Bioengineering
Ruth L. Kirschstein National Research Service Awards (NRSA)

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Searle Scholars Program
Sherman Fairchild Foundation
Simons Foundation
Skirball Foundation
Swartz Foundation

Targacept, Inc.
Technology Transfer Grubstake Award
That Man May See, Inc.
Thomas Hartman Foundation
Thome Foundation

United States Department of Defense
University of California, Tobacco-Related Disease Research Program

Vanguard Charitable Endowment in memory of Bently Pritsker

WCU Program through the National Research Foundation of Korea
Weston Havens Foundation
Whitehall Foundation