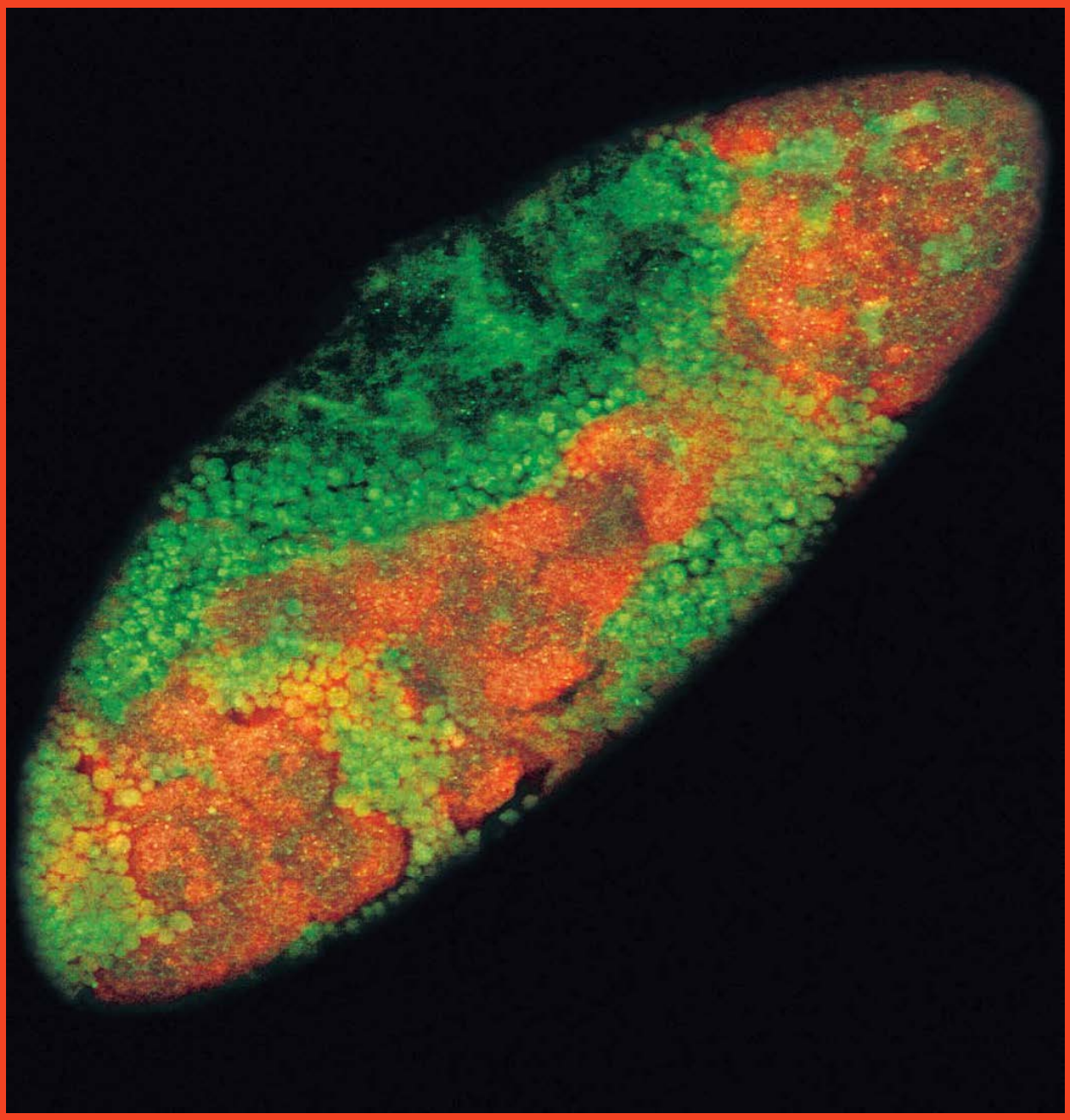

CALTECH Biology Annual Report 2002

FRONT COVER

Jun Huh and Associate Professor B.A. Hay

(Confocal image of the *Drosophila Melanogaster* embryo)

Expression of the *Drosophila Melanogaster* apoptosis inducer Hid promotes the ubiquitination and degradation of the caspase inhibitor Diap1 (red). Cells in which Diap1 is eliminated undergo caspase activation (green) and apoptosis. Embryos were immunohistochemically stained with Diap1 Ab and active specific Drice Ab.

BACK COVER

S. Murase, E. Mosser and Associate Professor E.M. Schuman

EGFP-β-catenin expressed in hippocampal neurons via sindbis virus infection. The fluorescent signal is concentrated in dendritic spines (post synaptic structure) where cadherin-β-catenin complex mediates synaptic connections with presynaptic terminals. The concentration of β-catenin in spines leads to an increase in both pre-and postsynaptic proteins and an increase in synaptic strength.

Division of Biology

California Institute of Technology

Pasadena, CA

Annual Report 2001 - 2002

BIOLOGY - 2002

Yolanda Duron, Annual Report Coordinator

I would like to express my sincere thanks to both Stephanie Canada and Tom Tubman for their assistance in the production of this report.

Research Reports

Biological research summarized in this report covers the time period from June, 2001, through July, 2002. The annual report is not intended to serve as an official forum, since some portions of the research listed in this report have not yet been published. When referring to an individual abstract(s), special permission must be obtained from the investigator.

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

MOLECULAR BIOLOGY AND BIOCHEMISTRY

CELLULAR, MOLECULAR AND DEVELOPMENTAL NEUROBIOLOGY

INTEGRATIVE NEUROBIOLOGY

Introduction

- 50 and 25 Years Ago -

Biology Annual Report 1952

"In recent years there has been a growing amount of evidence indicating that genes with closely related functions often lie physically close together in a chromosome…Several such complexes of genes, in particular the bithorax complex, have been studied in *Drosophila* during the past year…. important clues have been found concerning the way in which individual genes control the development of the thorax and the abdomen."

"The total amount spent by the Division of Biology on instruction and research in the fiscal year 1951-1952 was in excess of $500,000…"

Bio-peeps: News Items about Biology Division People, June 6, 1952

"The gastronomic word from the Sterling Emersons in France is that the Parisian cuisine, though expensive, lives up to its reputation… They reported meeting a number of former Kerckhoffians in Boris Ephrussi's laboratory at the University of Paris and at Andre Lwoff's at the Pasteur Institute. Among them were: Curt Stern, Jacques Monod, Eli Wollman, Seymour Benzer, Gunther Stent and Urs Leupold."

"The National Science Foundation has made six grants to Caltech totaling $73,700 for the support of research projects and Division of Biology Staff members. The investigators and grants were as follows:

James Bonner and Anton Lang, $17,700 for a two-year study of the physiology and biochemistry of flowering; James Bonner and George Laties, $10,500 for a one-year study of the biochemistry of plant growth; Max Delbrück and Marguerite Vogt, $5,500 for a one-year study of the mechanisms underlying genetic recombination in bacteria; A.W. Galston, $5,500 for a one-year study of auxin physiology; F.W. Went, $21,700 for a three-year study of physiological differences among races and varieties of higher plants; and, C.A.G. Wiersma, $13,300 for a two-year study of the central nervous system of lower animal forms."

Biology Annual Report 1977

"Professor Robert L. Sinsheimer, Professor of Biophysics since 1957 and Chairman of the Division since 1968, has left the Institute to become Chancellor of the University of California at Santa Cruz. He was succeeded on September 1 by the new chairman, Professor Norman Horowitz. Professor Horowitz, a geneticist and member of the National Academy of Sciences, was a graduate student in this Division (1936-1939) and has been a member of the faculty since 1947."

"We found that nearly full-length double-strand DNA copies of rabbit beta-globin mRNA could be synthesized in vitro using the combined activities of AMV reverse transcriptase, *E. coli* DNA polymerase I, and S1 nuclease (Efstratiadis *et al*., 1976), and that this DNA could be joined to a bacterial plasmid, cloned and amplified without sequence rearrangements (Maniatis *et al*., 1976)…" From the report of Associate Professor Thomas P. Maniatis.

"Ours is a small, generally happy research group, busy at the crossroad of mammalian genetics and immunology. The Professor has a lot of things to do (he is also Dean of Students and Vice President for Student Affairs at the Institute)…" From the report of Professor Ray D. Owen

Michael Dickinson

Grant Jensen

Thanos Siapas

Professor Michael Dickinson joined the Divisions of Biology and Engineering and Applied Sciences this year. His primary research interests concern the physiology and mechanics of flight behavior in insects. Specifically, he has focused on the flight control system of the flies--arguably the most aerodynamically sophisticated of all flying animals. His research strategy is to tackle flight behavior using approaches from such disparate disciplines as neurobiology, structural engineering and aerodynamics. Thus, in Professor Dickinson's lab they attempt to study flight control behavior simultaneously at several levels of analysis, from the physiological properties of individual neurons and circuits, to the skeletal mechanics of wing motion and the production of aerodynamic forces.

Professor Dickinson received his Sc.B. in Neural Sciences from Brown University and his Ph.D. in Zoology from the University of Washington. Before joining Caltech, he was the Williams Professor of Integrative Biology at the University of California, Berkeley.

Professor Dickinson is also member of the Society for Neuroscience, International Society for Neuroethology and Society of Integrative and Comparative Biology.

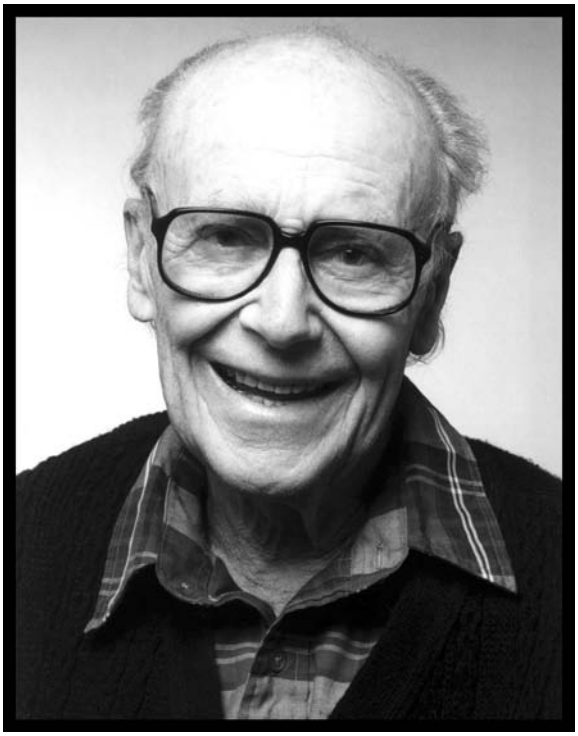
Professor Grant Jensen joined the Division of Biology faculty in August of this year. His research focuses upon developing the techniques of cryoelectron microscopy to examine the three dimensional structures of large macromolecular machines and cells. For these studies, he uses electron tomography, which refers to a 3D reconstruction derived from images corresponding to 2D projections of an object such as a virus or intact cell. Professor Jensen has initiated projects to apply electron tomography to the studies of a single cell (*Mycoplasma genitalium*) and a virus (HIV). In addition, he has developed a new approach for aligning molecules in single particle analysis, which should allow higher resolution images of macromolecular assemblages such as the proteasome or viruses to be obtained by electron tomography. Professor Jensen's research interests also include whole cell computer simulations of the structure and dynamics of organisms, based on the three-dimensional spatial organizations of those cells derived from his electron microscopy studies.

Professor Jensen received a B.S. in Physics from Brigham Young University and a Ph.D. in Biophysics from Stanford University. Before joining the Caltech faculty, he was a postdoctoral fellow in Kenneth Downing's laboratory at the Lawrence Berkeley Laboratories.

Professor Thanos Siapas arrived on campus this past January. His research focuses on the study of the principles underlying learning and memory formation across distributed networks of neurons. Using techniques that allow monitoring of simultaneous activity of dozens of single neurons in freely behaving animals, he studies the structure of interactions between the hippocampus and neocortical brain areas and the role of these interactions in learning and memory. He also investigates the cellular and molecular basis of network interactions by analyzing the effects of pharmacological and genetic manipulations on the organization of ensemble neuronal activity. His experimental work is complemented by theoretical studies of network models and the development of tools for the analysis of multi-neuronal data.

Professor Siapas received a B.S. in Mathematics, a B.S. in Electrical Science and Engineering, an M.S. in Electrical Engineering and Computer Science, and a Ph.D. in Electrical Engineering and Computer Science, from the Massachusetts Institute of Technology. Before joining Caltech, he conducted postdoctoral research in behavioral electrophysiology in Matt Wilson's laboratory at MIT.

Professor Siapas has a joint appointment in Caltech's Division of Biology and Engineering and Applied Sciences.



Norman Chandler Professor of Chemical Biology, Emeritus Norman Davidson died on February 14, 2002, after a brief illness. He was 85. He had been a Caltech faculty member since 1946, first in the Division of Chemistry and Chemical Engineering, then in Biology. He took emeritus status in 1986, but served as executive officer for biology from 1989 to 1997 and remained active in research until his death.

Davidson was born April 5, 1916, in Chicago. He earned a bachelor's degree in chemistry at the University of Chicago in 1937, and completed another Bachelor of Science degree at the University of Oxford in 1939 as a Rhodes Scholar. In 1941 he completed his doctorate in chemistry at the University of Chicago.

During the war he worked for the National Defense Research Committee Project at the University of Southern California, and for the Division of War Research at both Columbia University and the University of Chicago. From 1943 to 1945, he worked in the University of Chicago's metallurgical laboratory on the Plutonium Project.

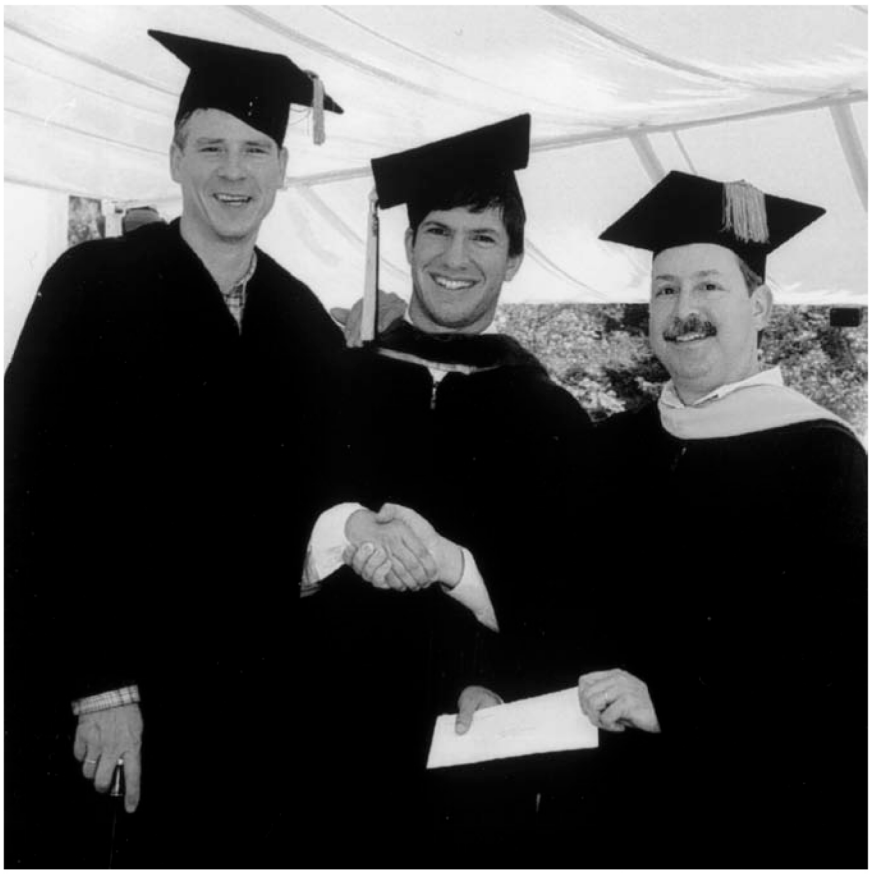
After the war and a brief stint as a researcher at the Radio Corporation of America, Davidson joined the Caltech faculty as a chemistry instructor, and remained on the faculty for the rest of his life. He became a tenured professor of chemistry in 1952; a full professor in 1957; executive officer for chemistry in 1967; and Norman Chandler Professor of Chemical Biology in 1982. He also served briefly as interim chair of the Division of Biology in 1989.

Davidson was known for his innovative methods in bridging the gap between the physical and biological sciences. He pioneered new methods in physical chemistry and electron microscopy, the latter proving especially useful for genetic mapping and exploring the informational properties of DNA and RNA.

In 1996, when he was awarded the National Medal of Science by President Clinton, Davidson was working on new methods for studying electrical signaling in the nervous system and the ways in which the system changes during learning and memory formation. He was cited by the White House "for breakthroughs in chemistry and biology which have led to the earliest understanding of the overall structure of genomes." "For example," the White House statement continued, "Davidson's research on DNA established the principle of nucleic acid renaturation, one of the most important principles in molecular biology and a primary tool for deciphering the structure and function of genes."

Davidson's many awards included his designation as the 1980 California Scientist of the Year; the Robert A. Welch Award in Chemistry (1989); the Dickson Prize for Science (1985); and the Peter Debye Award by the American Chemical Society (1971). He was a member of the National Academy of Sciences for 42 years, a fellow of the American Academy of Arts and Sciences since 1984, and held an honorary doctorate from the University of Chicago.

HONORS AND AWARDS - 2002



Koch Kreiman Meyerowitz

The Milton and Francis Clauser Doctoral Prize for the most original Ph.D. thesis at Caltech, and the **Lawrence L. and Audrey W. Ferguson Prize** for the most outstanding Ph.D. thesis in Biology were both awarded to Gabriel Kreiman for his dissertation, "On the neuronal activity in the human brain during visual recognition, imagery and binocular rivalry" in June, 2002. Gabriel's research in the lab of Professor Christof Koch deals with the relationship between the flow of images that we experience in our daily life and the neural activity in our brains that must give rise to these subjective states. Working under the clinical supervision of the neurosurgeon Dr. Itzhak Fried at the UCLA School of Medicine, Gabriel took advantage of a unique opportunity to record the electrical signals of individual nerve cells in the medial temporal lobe of epileptic patients implanted with depth electrodes to localize the focus of seizure onset. Gabriel found that many neurons respond to individual images, such as a particular face, or to categories of images, such as animals. He discovered a class of cells that responds either when the patient sees a picture of a dolphin or is asked to imagine a dolphin. That is, the mechanism of visual imagery is closely related to the mechanism of normal seeing. Gabriel also characterized neuronal responses to a visual illusion that reveals that these cells closely follow the subjective, conscious percept rather than the objective, retinal stimulus. Simultaneous with his PhD in Biology, Gabriel also obtained a MS degree in CNS.

Graduate Deans' Award for Outstanding Community Service
Both Alejandro Bäcker (Biology) and Teerachai N. Pornsinsirirak (Electrical Engineering) received the 2002 award which is awarded to a Ph.D. candidate who, throughout their graduate years at the Institute, have made great contributions to graduate life and whose qualities of leadership and responsibility have been outstanding.

PROFESSORIAL AWARDS 2002

James G. Boswell Professor of Neuroscience Richard Andersen was elected to the American Academy of Arts and Sciences.

Professor of Biology David J. Anderson was elected to the American Academy of Arts and Sciences in April, 2002.

James G. Boswell Professor of Neuroscience Professor Seymour Benzer, Emeritus (active), was awarded the Pasarow Award for Medical Research in Neuroscience; the March of Dimes Award in Developmental Biology (with Sydney Brenner); and received the National Academy of Sciences Award in the Neurosciences.

Professor Pamela Bjorkman, Full Investigator-HHMI, was elected as member of the American Philosophical Society.

Albert Billings Ruddock Professor of Biology Marianne Bronner-Fraser - NASA Life Sciences Panel for Developmental Biology (1999-present); Chair of the Faculty, Caltech, 2001-2003; Javits Award.

Allen and Lenabelle Davis Professor of Biology Mary Kennedy was elected to the American Academy of Arts and Sciences, and was given the Allen and Lenabelle Davis Professorship in Biology.

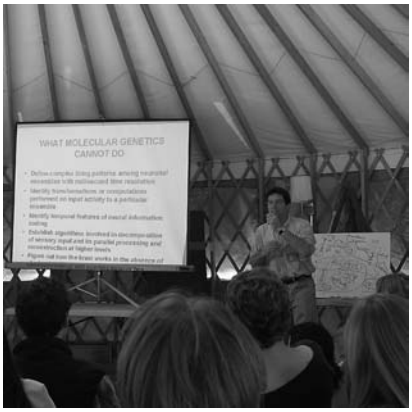
George W. Beadle Professor of Biology Elliot Meyerowitz was elected a Foreign Associate of the French Academy of Sciences in 2002. He was in June, 2001, the recipient of a European Flying Fellowship in Plant Molecular Biology, as part of which he gave a series of lectures at the University of Wageningen in the Netherlands, the University of Gent in Belgium, the Institut des Sciences Végétales in Gif-sur-Yvette, France, and the Max-Planck-Institut für Züchtungsforschung in Köln, Germany. He also gave the Annual Biology Lecture at the Swiss Federal Institute of Technology in Zürich on November 7, 2001.

Howard and Gwen Laurie Smits Professor of Cell Biology Alexander Varshavsky has received the 2002 Wilson Medal from the American Society for Cell Biology. He shared this award with Avram Hershko (Technion, Haifa, Israel). In 2002, Varshavsky gave the Dean's Lecture at the Mount Sinai School of Medicine (New York), University Lecture at the Southwestern Medical Center (Dallas), and the Chiron Lecture at the University of California, Berkeley.

NeuroBiology Retreat Group Photo, El Capitan Canyon, May, 2002











Photos from NeuroBiology Retreat taken at El Capitan Canyon weekend of May 10, 11, and 12, 2002
Photos provided by Elizabeth Chiang and Pietro Perona

Photos from Broad Center dedication on September 10, 2002

Photos provided by Herb Shoebridge

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Biology

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Biology

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Biology

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Biology

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Biology

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Biology

[1]*Joint appointment with Howard Hughes Medical Institute*
[2]*Undergraduate Student Advisor*

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Biology

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Biology

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Biology

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

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Biology

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Lisa R. Girard - Ph.D.
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Lingjie Gu - Ph.D.
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Rong-Gui Hu - Ph.D.
Byung Joon Hwang - Ph.D.[1]
Jong-Ik Hwang - Ph.D.

Takao Inoue - Ph.D.
Toshiro Ito - Ph.D.

Chung-Jiuan Jeng - Ph.D.
Mili Jeon - Ph.D.
Seong-Yun Jeong - Ph.D.[1]
Changan Jiang - Ph.D.
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Jaesang Kim - Ph.D.[1]
Soo-Mi Kim - Ph.D.[1]
Anne K. Knecht - Ph.D.
Irene Knuesel - Ph.D.
Reinhard Koester - Ph.D.
David Koos - Ph.D.
Takumi Koshiba - Ph.D.
Abraham Kovoor - Ph.D.

Postdoctoral Scholars

Michelene N. Laurent - Ph.D.
Tim Lebestky - Ph.D.
Joon Lee - Ph.D.[1]
Vivian Lee - Ph.D.
John F. Leite - Ph.D.
Daniela Leopoldt - Ph.D.
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Noltan Nadasdy - Ph.D.
Raad Nashmi - Ph.D.

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Nina T. Sherwood - Ph.D.
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W. Daniel Tracey Jr. - Ph.D.
Glenn C. Turner - Ph.D.

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Horng-Dar Wang - Ph.D.
Jianghai Wang - Ph.D.[1]
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Hae Yong Yoo - Ph.D.[1]
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Tzu-ping Yu - Ph.D.
Mary Yui - Ph.D.

Jin Zhang - Ph.D.
Yuanxiang (Ansel) Zhao - Ph.D.
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Jianmin Zhou - Ph.D.
Eva C. Ziegelhoffer - Ph.D.
Lisa Taneyhill-Ziemer - Ph.D.
Mark J. Zylka - Ph.D.

[1]Joint appointment with Howard Hughes Medical Institute

Visitors

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Leonard D. Bogarad - Ph.D.
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Andres Collazo - Ph.D.
Alberto Erives - Ph.D.
Andrew K. Groves - Ph.D.
Rex A. Moats - Ph.D.
Nicola Raule - B.S.
Ruffin Van Rullen - Ph.D.
Ying You - Ph.D.

Graduate Students

Aakalu, Girish
Adams, Meghan
Alvizo, Oscar[5]
Ambroggio, Xavier
Arthur, Ben[1]
Azzam, Ramzi

Backer, Alex
Bak, Magdalena
Baker, Catherine
Barbee, Susannah
Basch, Martin
Bender, John
Benjamin, Jordan
Bernheim, Kyle
Bhattacharyya, Sujata
Bingol, Baris
Bolon, Daniel[5]
Brandt, Gabriel[2]
Broome, Bede
Brown, C. Titus
Bui, Yen
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Bush, Eliot

Carter, Ronald M.
Cassenaer, Stijn
Chen,Tianxin[5]
Choe, Wonchae
Choi, Eun Jung
Choi, Gloria
Cope, Gregory[5]
Copeland, Jeffrey
Croal, Laura

Datta, Deepshikha[5]
Detmer, Scott
Dionne, Christopher
Du, Fangyong
Dubowitz, David[1]

Edwards, Jessica
Ewald, Andrew[5]

Farivar, Shabnam S.
Fernandes, Jolene

Gerety, Sebastian
Ghaboosi, Nazli
Giannetti, Anthony[5]
Gold, Daniel
Gong, Ying[5]
Green, Harry
Graumann, Johannes
Griffin, Erik

Hamburger, Zsuzsa
Hart, Christopher
Hemmati, Houman
Hidalgo, Loretta
Hochstim, Christian
Hom, Geoffrey[5]
Huang, Jean
Huang, Po-ssu[5]
Huh, Jun Ryul

Kim, Jongmin
Kirouac, Martha
Kreiman, Gabriel

Lassila, Kirsten[5]
Lee, Brian
Lee, Pei Yun
Leonardo, Anthony[1]
Leung, Thomas H.C.
Lin, Ben
Livi, Carolina
Lu, Carole

Mah, Angie[5]
Malasarn, Davin
Marshall, Shannon[2]
Medina-Marino, Andrew
Meulemans, Daniel
Mo, Chunhui[5]
Montgomery, Jennifer
Moreno, Tanya
Mosser, Eric
Muffat, Julien

Pesaran, Bijan[4]
Pesavento, Alberto[3]

Quinonez, Carlo

Ramirez-Lugo, Juan
Rosen, Tobias
Rosenbluth, David[1]

Sakamoto, Kathleen - M.D.
Salazar, Anna
Shah, Premal[5]
Shin, Dong Hun
Simion, Claudiu
Simon, Jasper
Slimko, Eric[1]
Smith, William B.
Southwell, Amber
Stanford, Theron[3]
Strop, Pavel[5]

Takahashi, Terry[5]
Tesar, Devin
Thorsen, Todd[5]
Tse, Eric

Vazquez, Luis
Voigt, Chris[5]

Yeager-Vernooy, Stephanie

Warren, Luigi
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Wiezorek, Jeffrey - M.D.
Wu, Daw-An

Yang, Lili
Yu, Hui

Zarnegar, Mark
Zhou, Qiao
Zid, Brian
Zirlinger, Mariela
Zollars, Eric[5]

Computational and Neural Systems[1]
Division of Chemistry and Chemical Engineering[2]
Division of Electrical Engineering[3]
Division of Engineering and Applied Physics[4]
Division of Biochemistry[5]

Biology Research and Laboratory Staff

Abramian, Armen - B.S.
Adams, Megan - B.S.
Alex, Jennifer M. - A.A.
Allen, Carolyn - M.A.
Amaya, Armando
An, Jee Young - B.S.
Andersen, Kristen
Andersen, Michael
Añonuevo, Lilia
Arias, Alexandra - B.S.
Arias-Robles, Denise
Armand, Elena - M.D.
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Ary, Marie - Ph.D.
Ausing, Andrej - B.S.

Babaoglan, Aysin B. - B.S.
Bae, Esther - B.A.
Balagot, Carlzen
Ballister, Edward
Barghshoon, Sami - B.S.
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Beel, Christie - B.A.
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Biondi, Ted - B.S.
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Boghosian, Maral - B.S.
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Boss, Shannan - B.S.
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Breznan, Boris - Ph.D.
Brothers, Alan - M.S.
Bugga, Lakshimi - M.S.

Campos, Ana
Cao, Yun - B.S.
Caron, Jennifer
Carpenter, Jeffrey D.
Carpenter, John - B.S.
Carrick, Deanna
Caton, Amy
Chan, Elisa Ya Kee
Chan, Juancarlos - B.S.
Chang, Jung Sook - B.S.
Chang, Mi-Sook - M.S.
Chee-Ruiter, Christine - Ph.D.
Chen, Yu-Jiun - M.S.
Chen, Wen - Ph.D.
Chetverikova, Eleonora
Chiang, Elizabeth - B.S.
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Choi, Johnny
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Colon, Ana
Condie, Robin
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Cronin, Christopher - B.S., M.E.
Csontos, Susanne

Damle, Sagar - B.S.
Darcy, Daniel - B.A.
Dasgupta, Chiranjib - Ph.D.
Delker, Sylvia - B.S.
Demodena, John - B.S.
Demyanenko, Tatyana - Ph.D.
Denny, Elisa - B.A.
Deshpande, Purnima - M.S.
Ding, Sheng
Dinh, Anh - B.S.
Dinh, Chau Bao - (KLM)
Dong, Ping - M.D.
Dowsett, Andrew - Ph.D.
Dunipace, Leslie - M.A.
Dunnick, Alejandro - B.S.
Duraj, Kevin - B.S.

Edens, Jean

Farkas, Maria - B.A.
Fish, Jennifer - B.S.
Flowers, Mary D. - M.A.

Gabbiani, Fabrizio
Gamboa, Jessica
Gause, Cheryl
Gharib, Shahla - Ph.D.
Gillikin, Betty - A.A.
Goard, Michael P.
Gomez, Amparo - B.S.
Gomez, Gustavo - B.S.
Gomez, Martha
Gonzalez, Constanza
Gonzalez, Jose
Granados, Blanca
Granados, Hernan
Gwirtz, Richele - B.S.

Hadju-Cronin, Yvonne
Hahn, Julie - B.S.
Hakeem, Atiya - Ph.D.
Hammond, Mark
Han, Jing - M.D.
Haraguchi, Erika
Hart, Rebecca - B.S.
Hartsteen, Parvin
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Hernandez, Carlos
Heyman, Karen - B.A.
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Hopkins, Sherrie Lee
Huang, Qi - B.S.
Huey-Tubman, Kathryn - M.S.

Itti, Laurent

Johnson, Jennifer
Jones, Cynthia
Jones, Matthew
Ju, Dong Hong - M.S.

Kairs, Myka J.
Kang, Joon - B.S.
Kato, Joyce - B.S.
Keeter, Aura
Kelsch, Carolyn
Kennedy, Bruce - M.S.
Kenny, Eimear - M.Sc., B.A.
Kewley, David - M.S.
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Kim, Young Mee - M.M.
King, Brandon
Kishore, Ranjana
Ko, Jan - B.S.
Koen, Patrick
Kostenko, Kyra - M.S.
Kou, Shi-Ying - M.D.
Kremers, David
Kulesa, Paul - Ph.D.

Laparra, Santiago
Lawrence, Nicholas - M.A.
Le, Niñon - B.S.
Leahy, Patrick - B.S. - (KLM)
Lee, Raymond - Ph.D.
Lee, Sun Young - M.S.
Lee, Vivian M.
Lenches, Edith M. - B.S.
Lim, Ha Yong - B.S.
Lin, Catherine - M.S.
Lisnock, Christine - B.S.
Liu, Jamie - B.S.
Llamas, Linda
Lo, Li-Ching - M.S.
Lo, Jr., Thomas - B.S.
Lorico, Arnulfo - B.S.
Lust, Ana Marie - B.A.
Lytle, Lloyd - B.S.

Macenka, Josephine - B.S.
Macias, Judy - A.A.
Malek, Christopher - B.S.
Mancino, Valeria, - M.S.
Mancuso, Gina - B.A.

Biology Research and Laboratory Staff

Mariona, Blanca
Marks, Cierina - A.A.
Marquina, Aurora
Marsh, Steven R. - B.A.
Mata, Jorge
Mata, Jose
Mathog, David R. - Ph.D.
McDowell, Doreen - M.S.
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Meda, Alfonso
Medina, Gladys
Medrano, Leonard J. - B.S.
Menon, Kaushiki - Ph.D.
Merchant, Edriss - B.S.
Mi, Qingli M.S.
Mosconi, Gabriella
Muñoz, Gustavo - B.A.
Muñoz, Jesse - A.A.

Nakamura, Cecilia
Nangiana, Jasvinder - B.S.
Nangiana, Inderjit - M.S.
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Negrillo, Alex - B.S.
Nesterova, Violana - M.S.
Newman, Laura - B.S.

Oania, Robert
O'Dell, Shannon
Oldark, Nick
Olsen, Corey
Olsen, John-Carl - M.S.
Ortega, Blanca

Page, Dolores - B.A.
Pant, Rashmi - Ph.D.
Park, Chang Sin - B.A.
Park, Sin Yee - M.S.
Perry, Barbara
Petcherski, Andrei - Ph.D.
Petrosyan, Agavni - B.A.
Phillips, Dianna - B.S.
Pillow, Rosetta - A.A.
Pinkney, Alan W. - B.A.
Pogodin, Timur - M.D.
Pogodina, Asya
Potter, Steven - Ph.D.

Quesada, Carole A.

Racs, John - B.S.
Rampersad-Ammons, Joanne
Rao, Anitha - M.S.
Rasche, Christoph
Rathbun, Alana
Rao, Anitha
Reis, Gerald - B.A.
Richards, Misty C.
Rigg, Jane - B.A.
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Santos, Nephi - A.B.
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Sendhil Velan, Sambasiyan - Ph.D.
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Shih, Cindy
Shilyanski, Carrie - B.S.
Shpak, Mitzi - B.S.
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Smith, Jeffrey O. - B.S.
Smith, Kayla - B.S.
Smith, Sue - M.A.
Solis, Diane
Speicher, Stephen - M.S.
St. James, Diana - B.A.
Story, Randall (KLM)
Supanyo, Kannika
Sutton, Jayne

Tan-Cabugao, Johanna
Tetreault, Nicole - B.S.
Thomas, Deanna
Thomas, Laurie A.
Thomas, Len - Ph.D.
Thomson, C. Ryan
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Wu, Peng - B.S.
Wu, Yan - M.S.
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Yamada, Joana - B.S.
Young, Carolina - B.S.
Young, Rosalind - M.S.
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Yu, Hong - M.S.
Yun, Gina - B.A.
Yun, Miki - B.A.

Zavzavadjian, Joelle - M.S.
Zedan, Rosario
Zimmerman, Ute - M.S.

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Scott Norton
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Hans Roggeband
Randall West

Cellular Biology and Biophysics

Charles J. Brokaw
Raymond J. Deshaies
William G. Dunphy

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. Most of the simulation programs, as Macintosh applications, are available at www.cco.caltech.edu/~brokawc/software.html

1. Coordination of dynein by local curvature control can generate helical bending waves

Charles J. Brokaw

Previous work with computer simulation of flagellar movement was performed with models that were restricted to bending in a plane. Computer simulations have been carried out now with a model flagellum that can bend in three dimensions. A pattern of dynein activation in which regions of dynein activity propagate along each doublet, with a phase shift of approximately 1/9 wavelength between adjacent doublets, will produce a helical bending wave. This pattern can be termed "doublet metachronism." The simulations show that doublet metachronism can arise spontaneously in a model axoneme in which activation of dyneins is controlled locally by the curvature of each outer doublet microtubule. In this model, dyneins operate both as sensors of curvature and as motors. Doublet metachronism and the chirality of the resulting helical bending pattern are regulated by the angular difference between the direction of the moment and sliding produced by dyneins on a doublet and the direction of the controlling curvature for that doublet.

A flagellum that is generating a helical bending wave experiences twisting moments when it moves against external viscous resistance. At high viscosities, helical bending will be significantly distorted by twist unless the twist resistance is greater than previously estimated. However, the mathematical methods used in the current models to calculate the effects of external viscous resistances, based on methods used for two dimensional modeling, are inadequate and provide only a crude approximation of the effects of external viscous resistances. These methods must be improved to fully understand the resistance to distortion by twist and to interpret experimental observations where high external viscosities cause transition from planar to helical bending.

Spontaneous doublet metachronism must be modified or overridden in order for a flagellum to generate the planar bending waves that are required for efficient propulsion of spermatozoa. Planar bending can be achieved with the three-dimensional flagellar model by appropriate specification of the direction of the controlling curvature for each doublet. However, experimental observations indicate that this "hard-wired" solution is not appropriate for real flagella.

Publications

Brokaw, C.J. (2001) Simulating the effects of fluid viscosity on the behavior of sperm flagella. *Math. Meth. Applied Sci.* **24**:1351-1365.

Brokaw, C.J. (2002) Computer simulation of flagellar movement VIII. Coordination of dynein by local curvature control can generate helical bending waves. *Cell Motil. Cytoskel*. **53**:103-124.

Associate Professor: Raymond J. Deshaies
Research Specialist I: Rati Verma
Research Fellows: Gabriela Alexandru, Rusty Lipford, Thibault Mayor, Dane Mohl, Matthew Petroski, Kathy Sakamoto[1]
Graduate Students: Xavier Ambroggio, Ramzi Azzam, Gregory Cope, Nazli Ghaboosi, Johannes Graumann, Angie Mah
Research and Laboratory Staff: Denise Arias-Robles, Robert Oania, Geoffrey Smith
[1]*UCLA STAR Program, Ph.D. candidate*

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American Cancer Society
Arnold and Mabel Beckman Foundation
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Summary: The Deshaies lab works on two basic biological phenomena: control of cell growth and division, and regulation of cell function by covalent attachment of ubiquitin or ubiquitin-like proteins to target polypeptides. Of particular interest to us is how attachment of ubiquitin to target polypeptides specifies their degradation, and how ubiquitin-dependent degradation is harnessed to regulate cell growth and division.

Defective control of cell growth and division can lead to disease. For example, inability to restrain cell proliferation can lead to cancer. Likewise, defects of the ubiquitin system have been linked to cancer and neurodegenerative diseases. Thus, understanding the mechanism of operation of the cell division machinery and the ubiquitin system will provide insight into diseases that affect millions of people.

We are using biochemical, molecular, and genetic approaches in both 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 cells and animal cells use essentially identical proteins to regulate basic cellular processes. Though we may not look the same to the untrained eye, we really are close cousins of the 'lowly' yeast!

Chromosome duplication and segregation are the key events underlying cell proliferation

For cells to proliferate, they must accurately duplicate their chromosomes, and distribute the duplicated chromosomes into two equal sets so that one set can be passed on to each daughter cell. The entire process of duplication and segregation is referred to as the cell cycle, and can be likened to a program.

Chromosome replication and segregation are very complex processes that involve hundreds of proteins. To help ensure that these proteins are mobilized to perform their tasks in a timely and orderly fashion, the cell cycle program employs control points that are referred to as 'transitions'. For example, the demarcation between the phase of the cell cycle program where cells rest between divisions (G1 phase) and the phase in which DNA replication occurs (S phase) is referred to as the G1->S transition.

Research in many other labs has shown that transitions are key decision-making points in the cell cycle program. For example, if a cell in G1 phase has damaged DNA, it pauses to repair the DNA before entering S phase, to ensure that the mutation is not propagated. In cancer, the cell cycle program proceeds through transitions in an unrestrained fashion, leading to uncontrolled cell duplication and the accumulation of chromosome damage that fuels the evolution of a cancer cell into a tumor. Thus, if we can understand how transitions work, and how they are regulated, we will gain important insight into how the cell cycle program works, and how it goes awry in cancer cells.

Two cell cycle transitions that are of interest to us are the G1->S and the mitosis->G1 transitions. At the G1->S transition, enzymes involved in the copying of DNA are switched on. At the mitosis->G1 transition, the microtubule spindle is disassembled and the dramatic changes in cell architecture that enable chromosome segregation are reversed to allow the cell to return to G1 phase. Over the past few years, my lab has focused on figuring out how the proteins involved in the G1->S and mitosis->G1 transitions work, and how their activities are controlled.

SCF and the G1->S transition

In our work on the G1->S transition in baker's yeast, we discovered an enzyme known as 'SCF ubiquitin ligase', whose job is to attach ubiquitin to other proteins. Ubiquitin typically serves as a molecular tag that marks proteins for destruction. One of the key targets of SCF is the protein Sic1. Sic1 restrains the G1->S transition by blocking the S phase-promoting activity of the S cyclin–cyclin-dependent kinase (Cdk) complex. At the end of G1 phase, SCF latches on to Sic1, and directs its modification with ubiquitin. A large hydrolytic chamber known as the proteasome then degrades the 'ubiquitinated' Sic1. In this manner, S cyclin-Cdk is emancipated from Sic1's inhibitory embrace and cells enter S phase and begin to copy their chromosomes. Surprisingly, although SCF was discovered in baker's yeast as an enzyme that controls the G1->S transition, it is now evident that there are many related SCF enzymes in yeast and human cells, and these enzymes control processes as diverse as embryonic development, immune response, and cell proliferation.

We continue to study how SCF works, how it is controlled, and how the proteasome destroys proteins ubiquitinated by SCF (see abstracts by M. Petroski and R. Verma). Insight into these topics will shed light into the basic cell biology of normal and diseased cells. We are also interested in how SCF is employed to regulate other cellular processes, including transcription (see abstract by R. Lipford). In addition to these basic studies, we have been developing a novel technology that exploits SCF activity to downregulate the activity of any desired protein in the cell (see abstract by K. Sakamoto). This technology may enable the development of new approaches for the identification and validation of drug targets, or even the design of new drugs.

Regulation of SCF by the ubiquitin-like protein Nedd8

Because of the important role that SCF plays in regulating cell behavior, we sought to identify proteins in the cell that bind SCF, and figure out how they might control its activity. This effort led us to identify the COP9 signalosome (CSN) as an important regulator of SCF. CSN is conserved throughout eukaryotes, but its function was unknown. Our studies revealed that CSN detaches a ubiquitin-like protein known as Nedd8 from the Cul1 component of SCF. Attachment of Nedd8 to Cul1stimulates SCF activity, and thus CSN may help shut off SCF activity. CSN has been implicated in a broad range of biological processes, including neuronal differentiation, axon guidance, photomorphogenesis in plants, and control of gene transcription. Our observations suggest that the diverse activities of CSN may arise from its ability to detach Nedd8 from Cul1 and related proteins. We are studying the structural basis of CSN action, and are attempting to identify substrate proteins (besides Cul1) that are acted upon by CSN (see abstracts by X. Ambroggio and G. Cope). Intriguingly, our preliminary data suggest that the Csn5 subunit of CSN contains a sequence motif (that we have dubbed the JAMM motif) that serves as the archetype for a family of putative metalloproteases that includes the Rpn11 subunit of the proteasome (see abstract by R. Verma). We are currently testing the hypothesis that other JAMM domain proteins are isopeptidases that detach ubiquitin or other ubiquitin-like proteins from substrates (see abstract by G. Alexandru).

Regulation of the exit from mitosis

As with the G1->S transition, the mitosis->G1 transition (also referred to as the exit from mitosis) is driven by ubiquitin-dependent proteolysis. The mitosis phase of the cell cycle program is dictated by an enzyme known as mitotic cyclin-Cdk (note that this enzyme is similar to that which promotes S phase, except that it contains mitotic cyclin instead of S phase cyclin). As long as mitotic cyclin-Cdk is on, the cell is in mitosis. At the end of mitosis, the mitotic cyclin component is degraded, which shuts off mitotic cyclin-Cdk activity, thereby allowing the cell to exit mitosis. The control of mitotic cyclin destruction is very complex. A protein known as Cdc14 switches on mitotic cyclin destruction. We previously demonstrated that, for most of the cell cycle program, Cdc14 is sequestered in an inactive state within a compartment of the cell termed the nucleolus. At the end of mitosis, Cdc14 is released from its nucleolar prison in an active form, and switches on an enzyme (APC ubiquitin ligase) that attaches ubiquitin to mitotic cyclin, triggering its degradation. We are now investigating the signals that specify Cdc14's brief furlough from the nucleolus to help us understand how the cell governs the activation of APC (see abstracts by R. Azzam, A. Mah, and D. Mohl).

Proteomics

Budding yeast - with its formidable arsenal of genetic, molecular genetic, biochemical, and cell biological techniques - is an ideal system in which to develop and test new approaches in proteomics. Currently, there are three proteomic-type projects in the lab. Johannes Graumann proposes to use a mutant construction technique devised by Alex Varshavsky ('ts degron') combined with mass spectrometry approaches developed in this lab, to investigate the heretofore unknown functions of a set of conserved, essential genes. Thibault Mayor's project is to devise new technologies for the identification and quantification of ubiquitin-conjugated proteins in crude cell extracts. Finally, Nazli Ghaboosi is generating tight, conditional mutations in the E1 enzyme of the ubiquitin pathway. These mutations will enable us to activate and deactivate ubiquitination by simply lowering or raising the temperature. Together, these projects will give us a comprehensive picture of the enzyme complexes in the cell that mediate ubiquitination, as well as their targets. Both of the first two projects will make use of an LCQ mass spectrometer that has just been acquired as part of a collaboration with the B. Wold laboratory.

2. Specificity and biological function of JAMM domain proteins

Gabriela M. Alexandru

The COP9 signalosome is a highly conserved multi-subunit protein complex, essential for development in multicellular organisms. Our group has previously shown that a proteolytic activity able to remove Nedd8 from its conjugates (deneddylase) is associated with the signalosome [Lyapina *et al*. (2001) *Science* **292**:1382-1385]. Furthermore, very recent data from our lab pointed to the Jab1/Csn5 subunit of the signalosome as being the deneddylase [Cope *et al.* (2002), submitted]. Sequence analysis revealed that Jab1 contains a metallo-isopeptidase motif, called JAMM for Jab1/MPN domain metalloenzyme that is essential for the deneddylase activity. The same motif is also present in the Rpn11 subunit of the proteasome, where it specifies a similar isopeptidase activity directed against ubiquitin conjugates [Verma *et al*. (2002), submitted]. Several other JAMM domain proteins have been identified in organisms ranging from archaea to humans. Current data outlined above suggests that these predicted isopeptidases could target Nedd8 or ubiquitin conjugates, but also any other ubiquitin-like protein conjugates. I plan to analyze the

specificity of different human JAMM domain proteins and to further use their known biochemical activity as a way to explore their biological function in mammalian cells.

3. Structural analysis of a novel metalloenzyme motif: The JAMM domain

Xavier Ambroggio

The Cop9 signalosome (CSN) is a multisubunit protein complex involved in numerous cellular processes. We have recently discovered that the CSN can promote the removal of the ubiquitin-like protein, Nedd8, from cellular proteins (Lyapina *et al*., 2001). We have found a novel motif, JAMM, for Jab1/MPN domain metalloenzyme motif, within the signalosome subunit Csn5 that is essential for this iso-peptidase activity *in vivo*. In an effort to establish evidence of a metalloprotease like fold within JAMM, we are determining the atomic crystal structure of a Jab1/Csn5 homolog. The three-dimensional structure of a Jab1/Csn5 homolog will provide a solid basis from which to dissect the various functions attributed to the protein.

4. The role of the mitotic exit network in the M/G1 transition

Ramzi Azzam

Exit from mitosis is an essential step in the progression of cells through the cell cycle. In late mitosis, inactivation of the mitotic cyclin/Cdk complexes causes mitotic spindle disassembly, chromosomal condensation, and return of cells to G1 phase. In budding yeast, this inactivation is either achieved by accumulation of a Cdk inhibitor (Sic1) or destruction of the cyclins by the anaphase-promoting complex (APC). Both of these mechanisms require a network of seven genetically interacting proteins that include three protein kinases (Cdc15, Cdc5, Dbf2), a protein phosphatase (Cdc14), a GTPase (Tem1), a GTP/GDP exchange protein (Let1), and a protein kinase regulator (Mob1). This group is collectively known as the mitotic exit network (MEN). We have recently proposed that the MEN controls mitotic exit by regulating Cdc14's localization. Cdc14 is sequestered in the nucleolus, tethered to Net1 and Sir2 in a complex known as the regulator of nucleolar silencing and telophase (RENT), throughout interphase. Upon activation of the MEN, Cdc14 is released from Net1, diffuses throughout the cell, and initiates cyclin proteolysis and the telophase-->G1 transition by dephosphorylating and thereby switching on the APC activator Hct1/Cdh1. In MEN mutants, Cdc14 remains in the nucleolus and these mutants fail to exit mitosis.

We are trying to deduce how the MEN controls the release of Cdc14 from RENT complex. One approach we undertook was to map *in vivo* phosphorylation sites on Net1 to determine how phosphorylation of Net1 influences Cdc14 release. Mutation of all 13 phosphorylated Ser and Thr residues to alanine initially showed no phenotype. Recently, in a report from the Amon group [Stegmeier *et al*. (2002) *Cell* **108**:207-220], the authors show that Cdc14 is transiently released from the nucleolus into the nucleus, and this early release depends on a protein network collectively termed the "FEAR" pathway named for (cdc **f**ourteen **ea**rly **r**elease). The MEN are now thought to maintain this FEAR-mediated release. Given the above observations, we examined and confirmed that the Net1 phosho-site mutants were defective in the FEAR-mediated release. Thus, our data indicate that the role of Net1 phosphorylation is to bring about the FEAR-mediated release of Cdc14. Further experiments are in progress to determine the exact phosphorylation sites required and to identify the kinase responsible for FEAR-dependent release.

5. Nedd8 protein modification and the COP9 signalosome

Gregory Cope

The COP9 signalosome (CSN) is a multi-subunit complex conserved from human to fission yeast *S. pombe*. This complex has been attributed to play a role in multiple processes, including photomorphogenesis in plants and cell cycle control in *S. pombe*. Given that all eight subunits of the CSN are highly homologous to the proteasome lid, a role in proteolysis has been proposed for the CSN. However the mechanism(s) underlying the true function of the CSN have yet to be elucidated.

We recently found that the CSN is associated with multiple cullin proteins (termed Cul1-5) in mammalian and yeast cells [Lyapina *et al*. (2001) *Science*, **292**:1382-1385]. Cullins are members of a class of E3 ubiquitin ligases that target specific substrates to ubiquitin dependent proteolysis. There are multiple ways in which cullins are regulated, one of which is through the covalent modification with the ubiquitin like protein Nedd8. This modification increases Cul1 ubiquitin ligase activity toward substrates *in vitro* and is essential in the fission yeast *S. pombe*. Interestingly, we found that CSN promotes the cleavage of the ubiquitin-like molecule Nedd8 from *S. pombe* Cul1 *in vitro* and *in vivo*. To elucidate the mechanism of this novel function, we undertook an investigation to identify the enzyme responsible for CSN dependent deneddylation. In this process, we have identified and characterized a putative metallo enzyme motif in Jab1/Csn5, which we term JAMM (Jab1/MPN domain associated metalloenzyme). Through genetic and biochemical analysis, we have found that JAMM is essential for Cul1 deneddylation and acts positively on Cul1 activity *in vivo*. We are currently trying to identify additional proteins that may be regulated by reversible cycles of neddylation/deneddylation mediated by Csn5.

6. Role of ubiquitin-activating enzyme in ubiquitin-dependent degradation

Nazli Ghaboosi

Ubiquitin-dependent proteolysis by the multi-subunit 26S proteasome is the major non-lysosomal pathway for protein degradation in eukaryotic cells. The pathway begins with the activation of ubiquitin by the E1 ubiquitin-activating enzyme. The ubiquitin moiety is transferred to one of several E2 ubiquitin-conjugating enzymes and is subsequently attached to the substrate, sometimes with the aid of an E3 ubiquitin ligase. The multi-ubiquitinated substrate is then targeted for degradation by the 26S proteasome through a poorly understood mechanism. It was originally thought that all substrates for the 26S proteasome must be ubiquitinated for proper targeting. However, there is convincing evidence that several proteins, notably ornithine decarboxylase and $p21^{Cip1}$, are targeted to the proteasome without ubiquitin modification. These ubiquitin-independent proteasomal substrates call into question the necessity of ubiquitination in proteasomal targeting as well as the mechanism of substrate-proteasome interactions.

We would like to characterize the role of ubiquitination in substrate targeting to the proteasome by identification of ubiquitin-independent substrates and ubiquitin-independent proteasome interacting proteins. While the list of known E2s, E3s, and substrates is steadily growing, there is only one E1 enzyme in all somatic eukaryotic cell types. At the apex of this intricate network, E1 offers a unique perspective from which to address the many unanswered questions regarding proteasomal targeting. Temperature-sensitive mutants of the essential yeast E1 gene, *UBA1*, have been created using PCR mutagenesis. These mutants will allow conditional disruption of the entire downstream ubiquitination pathway. The effect of *UBA1* mutations on ubiquitin-protein conjugation and protein degradation are being assayed. E1-independent proteasome substrates will be identified using quantitative proteomics to screen for short-lived proteins that are not stabilized in *uba1* mutant cells. Finally, the ubiquitin-dependence of proteasome-protein interactions will be determined by comparing mass spectrometric profiles of affinity-purified 26S proteasome interacting proteins from wild-type and *uba1* mutant cells.

7. Analysis of a set of unknown genes in the yeast *Saccharomyces cerevisiae*

Johannes Graumann

The completion of the genome of *Saccharomyces cerevisiae* revealed more than one third of the identified genes to be of unknown function. In the wake of the yeast genome sequencing project, the Saccharomyces Genome Deletion Project [Winzeler (1999) *Science* **285**:901] systematically deleted every open reading frame (ORF) in the yeast genome and identified about 750 essential genes.

Matching the genes of unknown function with those reported to be essential resulted in a list of about 180 open reading frames, which are essential and unknown. Blasting this list against the complete GenBank database revealed a set of 30 ORFs that are essential in yeast, highly conserved among eucaryota and which have no known functions.

The functional context of these candidates for novel biological mechanisms is being probed by immune purification through a double epitope tag [Seol (2001) *Nat. Cell Biol.* **3**:384] and the subsequent identification of co-purifying proteins by mass spectrometry based multidimensional protein identification technology [MuDPIT; Link (1999) *Nat. Biotech.* **17**:676]. A functional classification of the bait-set is being extrapolated from the annotation of associating proteins and will be further investigated.

8. Regulation of chromatin processes by ubiquitination and proteolysis

Rusty Lipford

Recent work of Yong Chi and others has established a link between ubiquitin-mediated proteolysis and transcriptional activation by Gcn4, an activator of amino acid biosynthetic genes. A component of the RNA polymerase II holoenzyme, Srb10, phosphorylates Gcn4 and targets the activator for ubiquitination and degradation. Interestingly, this process requires that Gcn4 be competent to bind DNA and to activate transcription. In addition, mutations in Gcn4 that eliminate Srb10 mediated phosphorylation stabilize the activator on the DNA, yet compromise its ability to activate transcription. We are currently investigating the nature of the transcriptional defect in the Gcn4 phosphorylation mutants. In addition we are examining the possibility that the ubiquitination and proteolysis machinery are recruited directly to promoters to regulate transcription. Similarly we are using genomic and proteomic analyses to uncover chromatin domains that may be regulated by ubiquitination and proteolysis.

9. The mitotic exit network in *S. cerevisiae*

Angie Mah, Ramzi Azzam

Exit from mitosis is ultimately triggered by the loss of cyclin-dependent kinase (Cdk) activity by degradation of mitotic cyclins and accumulation of Cdk inhibitors. Cdc14 plays a critical role in triggering this event as it dephosphorylates, thereby activating, the inhibitors of mitotic Cdk, activating the inhibitors, which in turn, inactivating the Cdk. Cdc14 activity is dependent on its localization, and genetic evidence has suggested that a regulatory group of proteins, the mitotic exit network (MEN), modulates its activity. The mitotic exit network consists of the group of genes TEM1, LTE1, CDC15, DBF2, DBF20, MOB1 and CDC5. Mutations in these genes have revealed similar phenotypes of arrest in late anaphase with separated chromosomes, an elongated spindle, and elevated Cdc28/Clb2 kinase activity. Genetic interactions with each other have also been shown, with Cdc14 as the endpoint of this pathway. Tem1 is a ras-like

GTPase, which is likely to be a target of Lte1, a guanine nucleotide exchange factor, whereas Cdc15, Dbf2, and Cdc5 are all protein kinases, while the function of Mob1 is still unknown.

In order to characterize this pathway, we have determined the functional interactions between Cdc15, Dbf2, and Mob1 by reconstituting the activation of Dbf2 using recombinant proteins expressed in the baculovirus system. We have demonstrated Cdc15 directly activates Dbf2, but only when Dbf2 is bound to Mob1. However, the substrate for the Dbf2-Mob1 kinase complex remains elusive. We are currently working on determining the optimal phosphorylation motif for Dbf2. By defining a consensus phosphorylation sequence, we hope to characterize Dbf2 substrate(s) that ultimately links the MEN pathway to its effector, Cdc14.

10. Global approaches for the identification of substrates of SCF ubiquitination pathways

Thibault Mayor

Proteins to be degraded by the ubiquitin-proteasome system are targeted by covalent conjugation of a multi-ubiquitin chain in a multi-step process called ubiquitination. The specificity of protein ubiquitination derives from the E3 ubiquitin protein ligases. Although some E3s are single proteins, many have been shown to be protein complexes. One particular class of E3s, referred to as Skp1-Cdc53/Cullin-F-box protein (SCF) complexes, uses particular adapter subunits called F-box proteins to recruit specific substrates for ubiquitination. A vast array of F-box proteins has been identified by interaction studies and from genome sequencing information. Whereas identification of the F-box protein family was facilitated by the presence of a sequence motif, only a few binding substrates have so far been isolated. Elucidation of these protein substrates remains, therefore, an important task that will uncover many other processes regulated by proteolysis.

We propose to exploit quantitative mass spectrometry method in order to identify protein substrates of the SCF ubiquitination pathway in *Saccharomyces cerevisiae*. Expression of specific F-box proteins will be modulated and global change of protein levels in the cell observed. The idea is that the absence of an F-box protein will prevent the degradation (and hence bring about the accumulation) of its specific protein substrates. After re-expression of the F-box protein, its substrates should be targeted for ubiquitination and degraded. Detection of changes in protein levels will be carried by mass spectrometry analysis.

11. Regulation of mitotic exit

Dane Mohl

Faithful segregation of chromosomes requires tight control over cytokinesis and exit from mitosis. In budding yeast, mitotic exit involves the proteolytic removal of B type cyclins (Clb) and concomitant accumulation of Sic1, a Clb/Cdk inhibitor. The mitotic exit network (MEN) links mitotic exit to DNA segregation, by regulating the release of Cdc14 from the RENT complex, where it is bound Net1. Cdc14 then dephosphorylates several targets, including Sic1 and Cdh1, that speed the elimination of Cdk1/Clb2 kinase activity. Release of Cdc14 from RENT appears to be regulated through the affinity of Net1 for Cdc14, and the transport of Cdc14 from the nucleus into the cytoplasm. In order to define the mechanisms that regulate Cdc14 localization, we are using live cell fluorescence microscopy and MUDPIT technologies (**m**ulti-**d**imensional mass spectrometric **p**rotein **i**dentification **t**ools) to investigate how the mitotic exit network controls the interaction of Cdc14 with both RENT, and the nuclear import/export machinery.

12. The mechanisms of substrate poly-ubiquitination and degradation

Matthew D. Petroski

The ubiquitin-proteasome pathway has emerged as a central regulatory mechanism of protein stability in eukaryotes. Through a series of energy-dependent enzymatic steps, a chain of the small protein known as ubiquitin is attached to a lysine residue of a protein, tagging the protein for degradation by the 26S proteasome. To investigate the mechanisms involved in this process, we have focused our efforts on the budding yeast cyclin dependent kinase (CDK) inhibitor Sic1 as we have previously developed an *in vitro* ubiquitination and degradation system that faithfully mimics *in vivo* turnover.

In vitro, all of the lysines of Sic1 can serve as acceptor sites for poly-ubiquitin attachment. However, it is clear that only a subset of these sites is actually important for *in vivo* turnover. We have determined that the assembly of Sic1 into a complex with S-phase CDK appears to physically shield certain lysines of Sic1, thereby defining the Sic1 degradation signal. Also, the removal of Sic1 from this complex appears to be the rate-limiting step in its degradation. Further studies are examining the relationship between the number of ubiquitin attachment sites with respect to protein turnover.

13. Targeting proteins for proteolysis by ubiquitin ligases: Potential therapeutic applications

Kathleen M. Sakamoto, M.D.[1]

A central goal of the pharmaceutical and biotechnology industries is to identify small, stable, cell-permeable molecules that inhibit the activity of cellular proteins that contribute to cancer. Despite the importance of identifying new protein inhibitors for use as therapeutics, there is no simple, straightforward strategy for doing so that works for all target proteins. We propose a general method for developing inhibitors that exploits the unique characteristics of the ubiquitin-dependent proteolytic system of eukaryotic cells. The ultimate goal of the research program proposed here will be to identify a cell-permeable molecule that binds to the substrate-

docking site of a ubiquitin ligase. By covalently linking this molecule to other compounds that bind specific cellular proteins, we intend to develop a novel class of drugs or ProTacs (Proteolysis Targeting Chimeric Pharmaceuticals) that can trigger the destruction of any protein in eukaryotic cells for which there exists a small, cell-permeable ligand. The goals of my project are to develop an experimental system to prove that this novel concept for drug design is experimentally feasible. At one end, Protac contains a peptide that binds with high affinity to the substrate-docking domain of the ubiquitin ligase b-TRCP. We then chemically linked the peptide to the fungal metabolite ovalicin, which binds covalently and specifically to the cellular enzyme methionine aminopeptidase-2 (MetAP-2). We have demonstrated as "proof of principle" that the resulting peptide-ovalicin Protac chimera is able to tether MetAP-2 to b-TRCP, and target MetAP-2 for ubiquitination and degradation.

To generalize this approach, we tested whether Protacs could target MetAP-2 to a different ubiquitin ligase. Cbl is a single polypeptide ubiquitin ligase that downregulates signaling molecules, including PDGF and EGF receptors. A peptide sequence of the T-cell receptor associating protein, Zap-70, was previously shown to bind to Cbl with high affinity. We chemically linked the zap-70 phosphopeptide to ovalicin to recruit Met-AP-2 to Cbl. Our results showed that we could target MetAP-2 for ubiquitination by Cbl *in vitro* using Protacs.

Our next challenge was to show that Protacs could recruit a different substrate to the $SCF^{b\text{-}TRCP}$ ubiquitin ligase for ubiquitination through non-covalent interactions. To this end, we tested the estrogen receptor (ER). A Protac was synthesized containing the IκBα phosphopeptide linked to the ligand estradiol. The estradiol- IκBα phosphopeptide Protac was able to recruit the ER to the $SCF^{b\text{-}TRCP}$, resulting in ubiquitination and degradation of ER. These experiments proved that Protacs can effectively target different substrates to ubiquitin ligases resulting in their destruction *in vitro*. We are presently testing the activity of Protacs in cells. Since the ER is known to promote proliferation of breast tumors, we hope to use Protacs to downregulate the ER and inhibit the growth of breast cancer cells.

[1]*Departments of Pediatrics and Cellular and Molecular Pathology, Division of Hematology-Oncology, Mattel Children's Hospital at UCLA, UCLA School of Medicine*

14. Determining the requirements for proteolysis by purified 26S proteasomes

Rati Verma

The 26S proteasome is the site of ubiquitin (Ub)- and ATP-dependent degradation of proteins involved in diverse regulatory pathways within the cell. Although Ub is recycled from proteins targeted for degradation, the molecular basis of substrate deubiquitination at the proteasome, and the role of this process in substrate degradation remain unknown. We have identified a novel deubiquitination activity associated with the Rpn11 subunit of the lid sub-complex of the 19S regulatory particle. Rpn11 contains a highly conserved Jab1/Pad1/MPN domain-associated metalloisopeptidase (JAMM) motif – $EX_nHXHX_{10}D$. Mutation of the predicted active site histidines to alanine (*rpn11AXA*) is lethal and stabilizes Ub-pathway substrates in yeast. $Rpn11^{AXA}$ assembles normally, and purified mutant proteasomes fail to deubiquitinate and degrade a ubiquitinated substrate *in vitro*. Our findings reveal a new class of deubiquitinating enzymes. They also suggest a unifying rationale for the presence of the lid sub-complex in eukaryotic but not prokaryotic proteasomes, and identify a new target for chemotherapeutic inhibition of proteasome activity.

Publications

Annan, R.S, Huddleston, M.J., Verma, R., Deshaies, R.J., and Carr, S.A. (2001) A multidimensional electrospray MS-based approach to phosphopeptide mapping. *Anal. Chem*. **73**:393-404.

Chen S.L., Huddleston, M.J., Shou, W., Deshaies, R.J., Annan, R.S. and Carr, S.A. (2002) Mass spectrometry-based methods for phosphorylation site mapping of hyperphosphorylated proteins applied to Net1, a regulator of exit from mitosis in yeast. *Mol. Cell. Prot.* **1**:186-96.

Chi, Y., Huddleston, M.J., Zhang, X., Young, R.A., Annan, R.S., Carr, S.A. and Deshaies, R.J. (2001) Negative regulation of Gcn4 and Msn2 transcription factors by Srb10 cyclin-dependent kinase. *Genes Dev*. **9**:1078-1092.

Deshaies, R.J. and Eilers, M. (2001) Editorial Review: Cell multiplication. *Curr. Opin. Cell Biol*. **13**:729-730.

Deshaies, R.J. and Ferrell, J.E. (2001) Multisite phosphorylation and the countdown to S phase. *Cell* **107**:819-822.

Deshaies, R.J., Seol, J.H., McDonald, W.H., Cope, G., Lyapina, S., Shevchenko, A., Shevchenko A., Verma, R. and Yates, J.R. (2002) Charting the protein complexome in yeast by mass spectrometry. *Mol. Cell. Prot*. **1**:3-10.

Galan, J.-M., Wiederkehr, A., Seol, J.H., Deshaies, R.J., Riezman, H. and Peter, M. (2001) Skp1p and the F-box protein Rcy1p form a non-SCF complex involved in recycling of the SNARE Snc1p in yeast. *Mol. Cell Biol.* **9**:3105-3117.

Lippincott J., Shannon K.B., Shou W., Deshaies R.J. and Li R. (2001) The Tem1 small GTPase controls actomyosin and septin dynamics during cytokinesis. *J. Cell Sci*. **7**:1379-1386.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A. and Deshaies, R.J. (2001) COP9 Signalosome promotes cleavage of NEDD8-CUL1 conjugates. *Science* **18**:1382-1385.

Mah, A.S., Jang, J. and Deshaies, R.J. (2001) Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex. *Proc. Natl. Acad. Sci. USA* **13**:7325-7330.

Sakamoto, K.M., Kim, K.B., Kumagai, A., Mercurio, F., Crews, C.C. and Deshaies, R.J. (2001) Protacs: Chimeric molecules that target proteins to the Skp1-Cullin-F box complex for ubiquitination and degradation. *Proc. Natl. Acad. Sci. USA,* Early Edition, **98**:8554-8559.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray W.M. Estelle M. and Deng, X.W. (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. *Science* **18**:1379-1382.

Seol, J.H., Shevchenko, A., Shevchenko, A. and Deshaies, R.J. (2001) Prospecting the Skp1-anchored proteome by mass spectrometry identifies RAVE, a regulator of V-ATPase assembly. *Nature Cell Biol.* **4**:384-391.

Shou, W. and Deshaies, R.J. (2002) Multiple telophase arrest bypassed (tab) mutants alleviate the essential requirement for Cdc15 in exit from mitosis in *S. cerevisiae*. *BMC Genet* 3,4.

Shou, W., Azzam, R., Chen, S.L., Huddleston, M.J., Baskerville, C., Charbonneau, H., Annan, R.S., Carr, S.A. and Deshaies, R.J. (2002) Cdc5 influences phosphorylation of Net1 and disassembly of the RENT complex. *BMC Mol. Biol.* 3,3.

Shou, W., Sakamoto, K.M., Keener, J., Morimoto, K.W., Traverso, E., Azzam, R., Hoppe, G.J., Feldman, R.M., DeModena, J., Moazed, D., Charbonneau, H., Nomura, M. and Deshaies, R.J. (2001) Net1 stimulates RNA polymerase I transcription and regulates nucleolar structure independently of controlling mitotic exit. *Mol. Cell* **8**:45-55.

Shou, W., Verma, R., Annan, R.S., Huddleston, M.J., Chen, S.L. and Deshaies R.J. (2002) Mapping phosphorylation sites in proteins by mass spectrometry. *Methods Enzymol*. **351**:279-296.

Traverso, E.E., Baskerville, C., Liu, Y., Shou, W., James, P., Deshaies, R.J. and Charbonneau, H. (2001) Characterization of the net1 cell cycle-dependent regulator of the Cdc14 phosphatase from budding yeast. *J. Biol. Chem*. **276**:21924-21931.

Verma, R., McDonald, W.H., Yates, J.R. III and Deshaies, R.J. (2001) Selective degradation of ubiquitinated Sic1 by purified 26S proteasome yields fully active S phase cyclin-Cdk. *Mol. Cell* **8**:439-448.

Wu, G., Lyapina, S., Das, I., Li, J., Guerney, M., Chui, I., Deshaies R.J. and Kitajewski, J. (2001) SEL-10, a negative regulator of notch signaling, targets notch for ubiquitin-mediated protein degradation. *Mol. Cell Biol.* **21**:7403-7410.

Zhou, C., Seibert, V., Geyer, R., Rhee, E., Lyapina, S., Cope, Greg., Deshaies R.J. and Wolf, D.A. (2001) The fission yeast COP9/signalosome is involved in cullin modification by ubiquitin-related Ned8p. *BMC Biochemistry* **2**:7.

Professor of Biology: William G. Dunphy
Senior Research Associate: Akiko Kumagai
Research Fellows: Seong-Yun Jeong, Soo-Mi Kim, Joon Lee, Wenhui Li, Jianghai Wang, Stephanie Yanow, Hae Yong Yoo
Graduate Students: Daniel Gold, Juan Ramirez-Lugo
Research and Laboratory Staff: Denise Arias-Robles, Esther Bae, Timur Pogodin

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Summary: In eukaryotic cells, the cyclin-dependent kinases (Cdks) control the progression of the cell cycle by regulating the accurate replication of the genome during S-phase and the faithful segregation of the chromosomes at mitosis (M-phase). The entry into these phases of the cell cycle is controlled by Cdks called S-phase promoting factor (SPF) and M-phase promoting factor (MPF). The action of these Cdks must be controlled both temporally and spatially in a very stringent manner. This strict regulation is imparted by a number of checkpoint mechanisms. For example, cells containing unreplicated DNA cannot enter mitosis due to the mobilization of the replication checkpoint. The Dunphy laboratory is engaged in the elucidation of the molecular mechanisms underlying the regulation of SPF and MPF during the cell cycle. Most of these experiments are conducted with *Xenopus* egg extracts, a system in which the entire cell cycle can be reconstituted *in vitro*.

The first member of the cyclin-dependent protein kinase family described is M-phase promoting factor (MPF), which contains the Cdc2 protein kinase and a regulatory subunit known as cyclin B. Since the identification of the molecular components of MPF, there has been rapid and extensive progress in unraveling the biochemistry of mitotic initiation. It is now well established that MPF acts by phosphorylating a myriad of structural and regulatory proteins that are involved directly in mitotic processes such as nuclear membrane disintegration, chromosome condensation, and mitotic spindle assembly. An ongoing challenge to the cell cycle field is the elucidation of how these phosphorylation reactions regulate the structural and functional properties of the various targets of MPF.

We have been most interested in how the cyclin-dependent protein kinases are regulated during the cell cycle. The principal focus of our laboratory has been on the regulatory mechanisms that govern the activation of MPF at the G2/M transition. Some immediate and long-term issues that we are tackling include:

1. What controls the timing of MPF activation so that it occurs at a defined interval following the completion of DNA replication?
2. How do various checkpoint or feedback controls influence the Cdc2/cyclin B complex?
3. What are the molecular differences between the simple biphasic cell cycle found in early embryonic cells and the more complex cell cycles that arise later in development?

More recently, we have been able to study at the molecular level some of the key events leading to the initiation of DNA replication at the G1/S transition. These events involve a cooperative interaction between the Origin Recognition Complex (ORC), the Cdc6 protein, and members of the Mcm family. These studies may ultimately help us understand how S-phase and M-phase are integrated with one another.

In principle, the regulation of cyclin-dependent kinases such as MPF could occur at any of several levels, including synthesis of the cyclin protein, association between the Cdc2 and cyclin proteins, or posttranslational modification of the Cdc2/cyclin complex. The posttranslational regulation of the Cdc2/cyclin complex is particularly important, even in early embryonic cells that manifest the simplest cell cycle programs. In recent years, many of the elaborate details of this Cdc2 modification process have been defined. For example, the binding of cyclin results in three phosphorylations of Cdc2: one at threonine 161 that is required for Cdc2 activity, and two dominantly inhibitory phosphorylations at threonine 14 and tyrosine 15. A variety of genetic and biochemical experiments have established that the inhibitory tyrosine phosphorylation of Cdc2 is an especially important mechanism of cell cycle regulation. As described in greater detail below, there is now strong evidence that the decision to enter mitosis involves considerably more than the tyrosine dephosphorylation of Cdc2. However, a thorough understanding of the kinase/phosphatase network that controls the phosphotyrosine content of Cdc2 will provide a firm foundation for understanding other facets of mitotic regulation.

Our laboratory has made substantial contributions to understanding the molecular mechanisms controlling the activation of the Cdc2 protein. For our studies, we utilize cell-free extracts from *Xenopus* eggs. Due to pioneering work in a number of the laboratories, it is now possible to re-create essentially all of the events of the cell cycle in these extracts. Consequently, it is feasible to study the molecular mechanisms of Cdc2 regulation in intricate detail with this experimental system. To facilitate these studies, we make extensive use of recombinant DNA technology to overproduce cell cycle proteins in either bacteria or baculovirus-infected insect cells. Moreover, in conjunction with our biochemical studies, we are taking advantage of the fission yeast system to exploit genetic approaches to identify novel *Xenopus* regulators of the cell cycle.

15. Coordination of S-phase with mitosis

Soo-Mi Kim, Stephanie Yanow, Juan Ramirez-Lugo

In eukaryotic cells, biochemical pathways have evolved to ensure that initiation of a late cell cycle event is dependent upon an early event. Particularly, entry into mitosis normally depends on successful completion of DNA replication. Perturbation of DNA synthesis by DNA

replication inhibitors or DNA damage arrests the cell cycle at a point termed the mitotic entry checkpoint. Mutants that disrupt the checkpoint control have been isolated from various organisms, suggesting that checkpoint control is due to an active mechanism rather than an intrinsic feature of mitosis itself. By using the *Xenopus* egg extract system, it has been shown that ongoing chromosome replication can induce the mitotic checkpoint, while inhibition of DNA replication by DNA polymerase inhibitors enhances this effect. Furthermore, DNA synthesis on artificial single-stranded DNA templates in S-phase extract is sufficient to delay mitosis, indicating that the DNA replication itself generates a signal triggering the arrest at S/M transition. Based on these observations, it is generally believed that the S-M checkpoint mechanism can sense a particular structure of DNA replication, e.g., protein complex assembled at the replication fork or the single-stranded DNA, and transmit a signal to the downstream cell cycle machinery. We are currently trying to employ a few different approaches to isolate the key players in the checkpoint pathway and to characterize their function in *Xenopus* egg extracts.

16. Regulation of *Xenopus* Plx1, a protein kinase that phosphorylates and activates the Cdc25 protein phosphatase

Hae Yong Yoo

The Cdc25 protein is a dual-specificity phosphatase that dephosphorylates both threonine-14 and tyrosine-15 on Cdc2 and activates the kinase activity of Cdc2 at the onset of mitosis. The Cdc25 protein has a highly conserved C-terminal catalytic domain and less conserved N-terminal regulatory domain rich in Ser-Pro and Thr-Pro motifs. The activity of the Cdc25 protein is regulated by phosphorylation. During mitosis, the N-terminal regulatory domain becomes highly phosphorylated on serine and threonine residues and the catalytic activity increases 5-10 fold. Many studies have indicated that both the Cdc2/cyclin B complex as well as an additional unidentified kinase phosphorylate Cdc25 at mitosis.

We set out to purify the unknown kinase responsible for activating Cdc25 from *Xenopus* extracts. This attempt was aided greatly by our finding that a major Cdc25-specific kinase distinct from Cdc2/cyclin B associates with the N-terminal domain of Cdc25. Using a *Xenopus* Cdc25 affinity column and several conventional chromatographic methods, we purified a Cdc25-specific kinase activity approximately 2500-fold. We isolated several micrograms of this protein (p67) and sequenced four of its tryptic peptides. PCR primers designed for two of these peptides were used to amplify a segment of the cDNA encoding p67. The PCR fragment was used to isolate a full-length 2.4 kb cDNA that contains all four tryptic peptide sequences. The amino acid sequence indicates that it is a typical Ser-Thr kinase with its catalytic domain in the N-terminal half of the polypeptide. It is clearly a member of the Polo family of protein kinases. Accordingly, we have named it Plx1, for Polo-like kinase from *Xenopus.* Recombinant six-histidine-tagged Plx1 was then produced in Sf9 insect cells and purified on nickel agarose. This kinase phosphorylates and activates Cdc25 *in vitro*. We are now studying the regulation of this enzyme, which may be a key regulator of mitosis in all eukaryotic cells.

17. The *Xenopus* Chk1 protein kinase mediates a caffeine-sensitive pathway of checkpoint control in cell-free extracts

Akiko Kumagai, Seong-Yun Jeong

The entry into mitosis is controlled by regulatory proteins that ensure the proper segregation of replicated chromosomes to daughter cells. The integrity of chromosomal DNA is under constant surveillance during the cell cycle. In eukaryotic cells, when chromosomes become damaged or cannot be replicated completely, mitosis is prevented by checkpoint mechanisms until two intact copies of the genome can be produced. A key target of these checkpoint pathways is the Cdc2-cyclin B complex, also known as maturation or M-phase promoting factor (MPF). The Cdc2-cyclin B complex, once activated at the G2/M transition, phosphorylates a myriad of proteins that carry out the various processes of mitosis such as nuclear disassembly and chromosome segregation.

In the presence of damaged or unreplicated DNA, Cdc2-cyclin B is kept inactive due to inhibitory phosphorylation of the Tyr-15 and Thr-14 residues of Cdc2. These phosphorylations are carried out collectively by the kinases Wee1 and Myt1. At the onset of mitosis, the phosphatase Cdc25C removes these inhibitory phosphate groups and thereby activates Cdc2-cyclin B. The activity of Cdc25C is strictly regulated, being low during interphase and high at mitosis. In recent studies in humans and *Xenopus*, it has been shown that Cdc25 is negatively regulated during interphase by the binding of 14-3-3 proteins. The inactive form of Cdc25 found prior to mitosis is phosphorylated on Ser-216 and Ser-287 in humans and *Xenopus*, respectively. This phosphorylation, which occurs in a consensus 14-3-3 binding site, mediates the association between Cdc25 and 14-3-3 proteins. This phosphorylation-dependent interaction appears to suppress the activation and/or action of Cdc25. Mutants of Cdc25 that cannot be phosphorylated at this residue and thus cannot bind 14-3-3 proteins override the unreplicated/damaged DNA checkpoint(s), suggesting that Cdc25 is a target of checkpoint regulation.

We have analyzed the role of the protein kinase Chk1 in checkpoint control by using cell-free extracts from *Xenopus* eggs. Recombinant *Xenopus* Chk1 (Xchk1) phosphorylates the mitotic inducer Cdc25 *in vitro* on multiple sites including Ser-287. The Xchk1-catalyzed phosphorylation of Cdc25 on Ser-287 is sufficient to confer the binding of 14-3-3 proteins. Egg extracts from which Xchk1 has been removed by immunodepletion are strongly but not totally compromised in their ability to undergo a cell cycle delay in response to the presence of unreplicated DNA. Cdc25 in Xchk1-depleted extracts remains bound to 14-3-3 due to the action of a distinct Ser-287-specific kinase in addition to Xchk1. Xchk1 is highly phosphorylated in the presence of unreplicated or damaged DNA, and this phosphorylation is abolished by caffeine, an agent that attenuates checkpoint control. The checkpoint response to unreplicated DNA in this system involves both caffeine-sensitive and caffeine-insensitive steps. Our

results indicate that caffeine disrupts the checkpoint pathway containing Xchk1.

18. Positive regulation of Wee1 by Chk1 and 14-3-3 proteins

Joon Lee

Wee1 inactivates the Cdc2-cyclin B complex during interphase by phosphorylating Cdc2 on Tyr-15. The activity of Wee1 is highly regulated during the cell cycle. In frog egg extracts, it has been established previously that *Xenopus* Wee1 (Xwee1) is present in a hypophosphorylated, active form during interphase and undergoes down-regulation by extensive phosphorylation at M-phase. We report that Xwee1 is also regulated by association with 14-3-3 proteins. Binding of 14-3-3 to Xwee1 occurs during interphase, but not M-phase, and requires phosphorylation of Xwee1 on Ser-549. A mutant of Xwee1 (S549A) that cannot bind 14-3-3 is substantially less active than wild-type Xwee1 in its ability to phosphorylate Cdc2. This mutation also affects the intranuclear distribution of Xwee1. In cell-free kinase assays, Xchk1 phosphorylates Xwee1 on Ser-549. The results of experiments in which Xwee1, Xchk1, or both were immunodepleted from *Xenopus* egg extracts suggested that these two enzymes are involved in a common pathway in the DNA replication checkpoint response. Replacement of endogenous Xwee1 with recombinant Xwee1-S549A in egg extracts attenuated the cell cycle delay induced by addition of excess recombinant Xchk1. Taken together, these results suggest that Xchk1 and 14-3-3 proteins act together as positive regulators of Xwee1.

19. Respective roles of Xchk1 and Xcds1 in checkpoint pathways

Zijian Guo, Wenhui Li, Jianghai Wang

In eukaryotic cells, biochemical pathways have evolved to ensure that initiation of a late cell cycle event is dependent upon an early event. Particularly, entry into mitosis normally depends on successful completion of DNA replication and of DNA repair. Perturbation of DNA synthesis or the presence of irreparable DNA damage arrests the cell cycle at a point termed the mitotic entry checkpoint. Mutants that disrupt the checkpoint control have been isolated from various organisms, indicating that the checkpoint control is due to an active mechanism involving kinase cascade signaling.

We have isolated one of the checkpoint kinases, *Xenopus* Cds1 (Xcds1), by using database analysis, polymerase chain reaction (PCR) amplification, and library screening. Xcds1 is phosphorylated and activated by the presence of some simple DNA molecules with double-stranded ends in cell-free *Xenopus* egg extracts. Xcds1 is not affected by aphidicolin, an agent that induces DNA replication blocks. In contrast, another checkpoint kinase *Xenopus* Chk1 (Xchk1) responds to DNA replication blocks, but not the presence of double-stranded DNA ends. Both Xcds1 and Xchk1 phosphorylate Cdc25 within a 14-3-3 binding site, which is required for a normal checkpoint response. Immunodepletion of Xcds1 (and/or Xchk1) from egg extracts does not attenuate the cell cycle delay induced by double-stranded DNA ends, suggesting that there are redundant mechanisms in this system that prevent mitosis in the presence of this type of DNA damage. Our findings indicate that in *Xenopus*, and perhaps other vertebrates, there are two pathways that respond to different kinds of signals from the DNA. Xcds1 and possibly another kinase(s) are activated by double-stranded DNA ends. In contrast, Xchk1 is unaffected by double-stranded DNA ends, but responds efficiently to replication blocks and UV damage, which also may act at least in part by perturbing replication.

20. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts

Zijian Guo

The checkpoint kinase Xchk1 becomes phosphorylated in *Xenopus* egg extracts in response to DNA replication blocks or UV-damaged DNA. Xchk1 is also required for the cell cycle delay that is induced by unreplicated or UV-damaged DNA. In this report, we have removed the *Xenopus* homolog of ATR (Xatr) from egg extracts by immunodepletion. In Xatr-depleted extracts, the checkpoint-associated phosphorylation of Xchk is abolished, and the cell cycle delay induced by replication blocks is strongly compromised. Xatr from egg extracts phosphorylated recombinant Xchk1 *in vitro*, but not a mutant form of Xchk1 (Xchk1-4AQ) containing nonphosphorylatable residues in its four conserved SQ/TQ motifs. Recombinant human ATR, but not a kinase-inactive mutant, phosphorylated the same sites in Xchk1. Furthermore, the Xchk1-4AQ mutant was found to be defective in mediating a checkpoint response in egg extracts. These findings suggest that Xchk1 is a functionally important target of Xatr during a checkpoint response to unreplicated or UV-damaged DNA.

21. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts

Akiko Kumagai, Joon Lee, Daniel Gold

We have identified Claspin, a novel protein that binds to *Xenopus* Chk1 (Xchk1). Binding of Claspin to Xchk1 is highly elevated in the presence of DNA templates that trigger a checkpoint arrest of the cell cycle in *Xenopus* egg extracts. Xchk1 becomes phosphorylated during a checkpoint response, and we demonstrate directly that this phosphorylation results in the activation of Xchk1. Immunodepletion of Claspin from egg extracts abolishes both the phosphorylation and activation of Xchk1. Furthermore, Claspin-depleted extracts are unable to arrest the cell cycle in response to DNA replication blocks. Taken together, these findings indicate that Claspin is an essential upstream regulator of Xchk1. We are currently analyzing various facets of the structure and function of Claspin.

22. Activation of Chk1 by mutagenesis of its TRF motif

Sophie Wang

Xenopus Chk1 (Xchk1) is required for the checkpoint-associated delay of the cell cycle in frog egg extracts containing unreplicated or UV-damaged DNA. Phosphorylation of Xchk1 at multiple sites in the SQ/TQ domain (residues 314-366) in response to unreplicated or UV-damaged DNA results in elevation of its kinase activity. We have found that mutagenesis of Thr-377 in the conserved Thr-Arg-Phe (TRF) motif of Xchk1 also leads to a substantial increase in kinase activity. Thr-377 does not appear to be a site of phosphorylation in Xchk1. These findings suggest that Thr-377 may play a role in suppressing the activity of Xchk1.

Publications

Budde, P.P., Kumagai, A., Dunphy, W.G. and Heald, R. (2001) Regulation of Op18 during spindle assembly in *Xenopus* egg extracts. *J. Cell Biol.* **153**:149-158.

Guo, Z. and Dunphy, W.G. (2000) Response of *Xenopus* Cds1 in cell-free extracts to DNA templates with double-stranded ends. *Mol. Biol. Cell* **11**:1535-1546.

Guo, Z., Kumagai, A., Wang, S.X. and Dunphy, W.G. (2000) Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in *Xenopus* egg extracts. *Genes & Dev.* **14**:2745-2756.

Kumagai, A. and Dunphy, W.G. (1999) Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. *Genes & Dev*. **13**:1067-1072.

Kumagai, A. and Dunphy, W.G. (2000) Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in *Xenopus* egg extracts. *Mol. Cell* **6**:839-849.

Lee, J., Kumagai, A. and Dunphy, W.G. (2001) Positive regulation of Wee1 by Chk1 and 14-3-3 proteins. *Mol. Biol. Cell* **12**:551-563.

Patra, D., Wang, S.X., Kumagai, A. and Dunphy, W.G. (1999) The *Xenopus* Suc1/Cks protein promotes the phosphorylation of G2/M regulators. *J. Biol. Chem.* **274**:36839-36842.

Wang, S.X. and Dunphy, W.G. (2000) Activation of *Xenopus* Chk1 by mutagenesis of threonine-377. *FEBS Letters* **487**:277-281.

Xia, Z., Morales, J.C., Dunphy, W.G. and Carpenter, P.B. (2001) Negative cell cycle regulation and DNA-damage inducible phosphorylation of the BRCT protein 53BP1. *J. Biol. Chem.* **276**:2708-2718.

Developmental Biology and Genetics

Marianne Bronner-Fraser
Eric H. Davidson
Scott F. Fraser
Bruce A. Hay
Edward B. Lewis
Elliot M. Meyerowitz
Ellen V. Rothenberg
Melvin I. Simon
Paul W. Sternberg
Alexander Varshavsky
Barbara J. Wold

Albert Billings Ruddock Professor of Biology: Marianne Bronner-Fraser
Member of the Professional Staff: Ben A. Murray
Visiting Associate: Mark A. J. Selleck
Visitors: Andrew K. Groves, Jack Sechrist
Senior Research Fellows: Sara C. Ahlgren, Clare V.H. Baker, Martin I. García-Castro
Postdoctoral Scholars: Meyer Barembaum, Maria Elena de Bellard, Laura Gammill, Yun Kee, Anne K. Knecht, Vivian Lee, Peter Lwigale, Kathryn McCabe, David W. McCauley, Lisa Taneyhill Ziemer
Graduate Students: Meghan Adams, Martin Basch, Sujata Bhattacharyya, Houman Hemmati, Daniel Meulemans, Tanya Moreno
Research and Laboratory Staff: Aysin Burcu Babaoglan, Joan Collazo, Mary Flowers, Gustavo Gomez, Jr., Constanza Gonzalez, Matthew Jones, Charles Mahoney, Anitha Rao, Nephi Santos, Jane Sutton, Johanna Tan-Cabugao
Undergraduates: Wendy Ching, Ronald Siu, Elizabeth Stameshkin

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Summary: This laboratory is concerned with analyzing the cellular and molecular events underlying the formation, cell lineage decisions and migration of neural crest cells. 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. As important precursors of the peripheral nervous system, neural crest cells have been of interest to the neuroscience community for decades. In addition to their specific neuronal and glial derivatives, neural crest cells can also form melanocytes, craniofacial bone and cartilage and smooth muscle. This plasticity makes the neural crest an exciting system in which to investigate the processes by which cells make decisions about their fate.

The neural crest is induced to form in the ectoderm at the junction between the neural plate and the prospective epidermis. Following neural tube closure, these cells leave the neural tube and migrate to diverse regions throughout the embryo. The pathways along which neural crest cells migrate are well characterized, and these cells are accessible to both surgical and molecular manipulations throughout their development. As a result, in addition to being an excellent system for investigating cell fate decisions, the neural crest has become an important model system for studying cell movement and behavior.

Our laboratory concentrates on studying the cellular and molecular mechanisms underlying the induction and early development of the neural crest. 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. These studies shed important light on the mechanisms of neural crest formation and migration.

Because neural crest cells 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.

23. Wnt in the ectoderm functions as a neural crest inducer

Martin Garcia-Castro, Christophe Marcelle, Marianne Bronner-Fraser

Neural crest cells, which generate most of the vertebrate peripheral nervous system and facial skeleton, arise at the border of the ectoderm and neural plate by an apparent inductive interaction between these tissues. Here, we report that Wnts function as neural crest inducers in the trunk region of avian embryos. Wnt6 has the proper distribution pattern in the ectoderm at the time of neural crest induction, and inhibition of Wnt signaling *in vivo* perturbs neural crest formation. Wnt molecules induce neural crest from naïve neural plate tissue *in vitro* a defined medium in the absence of added factors; in contrast, BMPs, which were previously proposed to induce neural crest, do so only in a complex medium. Taken together, these data suggest that Wnt molecules are both necessary and sufficient to induce neural crest cells in avian embryos.

24. Sonic hedgehog rescues cranial neural crest cell death induced by fetal alcohol syndrome

Sara Ahlgren, Vijaya Thakur, Marianne Bronner-Fraser

Alcohol is a teratogen that induces a variety of abnormalities, including brain/facial anomalies and reduced growth, with the exact nature of the defect depending upon the time and magnitude of the dose of ethanol to which developing fetuses are exposed. In addition to abnormal facial structures, ethanol-treated embryos exhibit a highly characteristic pattern of cell death. Dying cells are observed in the premigratory and migratory neural crest cells that normally populate most

facial structures. The observation that blocking Sonic hedgehog (Shh) signaling results in similar craniofacial abnormalities prompted us to examine whether there was a link between this aspect of fetal alcohol syndrome and loss of *Shh*. We demonstrate that administration of ethanol to chick embryos results in a loss of *Shh*, as well as a loss of transcripts involved in Shh signaling pathways. In contrast, other signaling molecules appear unchanged. Furthermore, we demonstrate that the cranial neural crest cell death induced by ethanol treatment can be averted by application of Shh. These data suggest that craniofacial anomalies resulting from maternal alcohol exposure are caused at least partially from loss of *Shh* and subsequent neural crest cell death.

25. Pax3-expressing trigeminal placode cells can localize to trunk neural crest sites but are committed to a cutaneous sensory neuron fate

Clare V.H. Baker, Michael R. Stark, Marianne Bronner-Fraser

The cutaneous sensory neurons of the ophthalmic lobe of the trigeminal ganglion are derived from two embryonic cell populations, the neural crest and the paired ophthalmic trigeminal (opV) placodes. Pax3 is the earliest known marker of opV placode ectoderm in the chick. Pax3 is also expressed transiently by neural crest cells as they emigrate from the neural tube, and it is re-expressed in neural crest cells as they condense to form dorsal root ganglia and certain cranial ganglia, including the trigeminal ganglion. Here, we examined whether $Pax3^+$ opV placode-derived cells behave like $Pax3^+$ neural crest cells when they are grafted into the trunk. $Pax3^+$ quail opV ectoderm cells associate with host neural crest migratory streams and form $Pax3^+$ neurons that populate the dorsal root and sympathetic ganglia, and several ectopic sites, including the ventral root. Pax3 expression is subsequently downregulated, and at E8, all opV ectoderm-derived neurons in all locations are large in diameter and virtually all express TrkB. At least some of these neurons project to the lateral region of the dorsal horn, and peripheral quail neurites are seen in the dermis, suggesting that they are cutaneous sensory neurons. Hence, although they are able to incorporate into neural crest-derived ganglia in the trunk, $Pax3^+$ opV ectoderm cells are committed to forming cutaneous sensory neurons, their normal fate in the trigeminal ganglion. In contrast, Pax3 is not expressed in neural crest-derived neurons in the dorsal root and trigeminal ganglia at any stage, suggesting either that Pax3 is expressed in glial cells or that it is completely downregulated before neuronal differentiation. Since Pax3 is maintained in opV placode-derived neurons for some considerable time after neuronal differentiation, these data suggest that Pax3 may play different roles in opV placode cells and neural crest cells.

26. Amphioxus and lamprey AP-2 genes: Implications for neural crest evolution and migration patterns

Daniel Meulemans, Marianne Bronner-Fraser

The neural crest is a uniquely vertebrate cell type present in the most basal vertebrates, but not in cephalochordates. Here, we study differences in regulation of the neural crest marker AP-2 across two evolutionary transitions—from invertebrate to vertebrate and from agnathan to gnathostome. Isolation and comparison of amphioxus, lamprey, and axolotl AP-2 reveals its extensive expansion in the vertebrate dorsal neural tube and pharyngeal arches, implying co-option of AP-2 genes by neural crest cells early in vertebrate evolution. Expression in non-neural ectoderm is a conserved feature in amphioxus and vertebrates, suggesting an ancient role for AP-2 genes in this tissue. There is also common expression in subsets of ventrolateral neurons in the anterior neural tube, consistent with a primitive role in brain development. Comparison of AP-2 expression in axolotl and lamprey suggests an elaboration of cranial neural crest patterning in gnathostomes. However, migration of AP-2 expressing neural crest cells medial to the pharyngeal arch mesoderm appears to be a primitive feature of vertebrates. Because AP-2 has essential roles in cranial neural crest differentiation and proliferation, the co-option of AP-2 by neural crest cells in the vertebrate lineage was a potentially critical event in vertebrate evolution.

27. Neural expression of mouse *Noelin-1/2* and comparison with other vertebrates

Tanya Moreno, Marianne Bronner-Fraser

Noelins are secreted glycoproteins with important developmental functions in frogs and birds. Here, we present the expression pattern of the mouse homolog of *Noelin-1/2* at E8-10 of development and compare this pattern to other vertebrates. Expression was observed in the neural plate and neural crest, as well as in the cranial ganglia. Later, expression is prominent in brain tissue and in the zone of polarizing activity (ZPA) in the limb.

28. Disruption of segmental neural crest migration and ephrin expression in Delta-1 null mice

Maria Elena De Bellard, Wendy Ching, Achim Gossler, Marianne Bronner-Fraser

Neural crest cells migrate segmentally through the rostral half of each trunk somite due to inhibition by ephrins in the caudal-half somites. To assess Notch/Delta function in this process, we examined neural crest migration and ephrin expression in Delta-1 mutant mice. Neural crest cells, identified by Sox-10 expression, migrated through both rostral- and caudal-half somites in mutants, consistent with a significant reduction in ephrinB2 levels in the somites. Later, mutant embryos have aberrantly fused and/or reduced peripheral ganglia, with a marked diminution in peripheral glia. These results show that Delta-1 is essential for proper migration and differentiation of neural crest cells.

29. Conservation of *pax* gene expression in ectodermal placodes of the lamprey

David W. McCauley, Marianne Bronner-Fraser

Ectodermal placodes contribute to the cranial ganglia and sense organs of the head and, together with neural crest cells, represent defining features of the vertebrate embryo. The identity of different placodes appears to be specified in part by the expression of different Pax genes, with Pax3/7 class genes being expressed in the trigeminal placode of mice, chick, frogs and fish, and Pax2/5/8 class genes expressed in the otic placode. Here, we present the cloning and expression pattern of lamprey Pax-7 and Pax-2, which mark the trigeminal and otic placodes, respectively, as well as other structures characteristic of vertebrate Pax genes. These results suggest conservation of Pax genes and placodal structures in basal and higher vertebrates.

30. Isolation of novel member of the spalt family of zinc finger genes in chickens that may be involved in neural crest cell fate determination

Meyer Barembaum, Marianne Bronner-Fraser

We have isolated a novel member of the spalt gene family, csal3, whose pattern of expression suggests that it may be involved in a number of embryonic processes including neural tube and branchial arch development. Spalt and spalt-related were first isolated in *Drosophila* as homeotoc genes. They have been shown to be involved in tracheal development and patterning of the wing veins, as well as regulating bristle formation through the regulation of pro-neural gene expression. The spalt gene family of zinc finger containing proteins is conserved in evolution: it has been isolated from *C. elegans* and a number of vertebrates including humans, fish and frogs. We have isolated a novel member of this gene family in chickens, csal3. It is expressed at the four somite-stage in the neural plate. As the neural tube closes its expression is lost in the dorsal neural tube. Later in neural development it is expressed in distinct regions of the brain and spinal chord. It is also expressed in the pharyngeal endoderm, but once the branchial arches form, csal3 expression is localized in the neural crest derived mesoderm of the arches. We can also detect csal3 in the lateral plate mesoderm and later in the distal limb bud. To determine its role in neural crest development we have electroporated a plasmid construct expressing csal3 and GFP into the neural tube of chicken embryos prior to neural crest emigration. Control embryos electroporated with the construct lacking the csal3 coding region contained GFP expressing cells throughout the trigeminal ganglia. In contrast, the embryos electroporated with the csal3-containing construct had very few if any GFP positive cells inside the ganglia, whereas most of the GFP positive cells at the level of the trigeminal ganglia were located outside the ganglia and did not express neuronal markers (Tuj1 or Hu). We are currently exploring how over-expression of csal3 affects the fate of the cranial neural crest cell.

Publications

Ahlgren, S. and Bronner-Fraser, M. (2002) Recycling signaling molecules during development. *Nature Neurosci.* **5**:87-88.

Ahlgren, S., Thakur, V. and Bronner-Fraser, M. (2002) Sonic hedgehog rescues neural crest cell death induced by fetal alcohol syndrome. *Proc. Natl. Acad. Sci. USA.* **99**:10476-10481.

Baker, C. and Bronner-Fraser, M. (2001) Vertebrate cranial placodes: I. Embryonic induction. *Dev. Biol.* **232**:1-61.

Baker, C., Stark, M. and Bronner-Fraser, M. (2002) Pax3-expressing ophthalmic trigeminal placode cells are committed to a cutaneous sensory neuron fate. *Dev. Biol.* **249**:219-236.

Bronner-Fraser, M. (2002) Molecular analysis of neural crest formation. *J. Physiol***. 96**:3-8.

DeBellard, M.E., Ching, W., Gossler, A. and Bronner-Fraser, M. (2002) Disruption of segmental neural crest migration and ephrin expression in Delta-1 null mice. *Dev. Biol.* **249**:121-130.

García-Castro, M., Marcelle, C. and Bronner-Fraser, M. (2002) Ectodermal Wnt function as a neural crest inducer. *Science* **297**:848-851.

Kee, Y. and Bronner-Fraser, M. (2001) Temporally and spatially restricted expression of the helix-loop-helix transcriptional regulator *Id1* during avian embryogenesis. *Mech. Dev*. **109**:331-335.

Kee, Y. and Bronner-Fraser, M. (2001) The transcriptional regulator *Id3* is expressed in cranial sensory placodes during early avian embryonic development. *Mech. Dev.* **109**:337-340.

Kee, Y. and Bronner-Fraser, M. (2001) *Id4* expression and its relationship to other Id genes during avian embryonic development. *Mech. Dev*. **109**:341-345.

Kious, B., Baker, C., Bronner-Fraser, M. and Knecht, A. (2002) Identification and characterization of a calcium channel gamma subunit expressed in differentiating neurons and myoblasts. *Dev. Biol.* **243**:249-259.

Knecht, A. and Bronner-Fraser, M. (2002) Induction of the neural crest: A multigene process. *Nature Rev. Gen.* **3**:453-461.

McCauley, D. and Bronner-Fraser, M. (2002) Conservation of Pax gene expression in ectodermal placodes of the lamprey. *Gene* **287**:129-139.

Meulemans, D. and Bronner-Fraser, M. (2002) Amphioxus and lamprey AP-2 genes: Implications for neural crest evolution and migration patterns. *Development.* In press.

Moreno, T. and Bronner-Fraser, M. (2001) The secreted glycoprotein *Noelin* promotes neurogenesis in *Xenopus*. *Dev. Biol*. **240**:340-360.

Moreno, T. and Bronner-Fraser, M. (2002) Neural expression of mouse Noelin-1/2 and comparison with other vertebrates. *Mech. Dev.* In press.

Norman Chandler Professor Cell Biology: Eric H. Davidson
Distinguished Carnegie Senior Research Associate Emeritus: Roy J. Britten
Visiting Associates: Hamid Bolouri[*], Leroy Hood[†], David R. McClay[‡]
Senior Research Associate: R. Andrew Cameron
Senior Research Fellows: César Arenas-Mena, Jonathan P. Rast, Chiou-Hwa Yuh
Member of the Professional Staff: Andrew Ransick
Research Fellows: Gabriel Amore, Cristina Calestani, Veronica Hinman, Takuya Minokawa, Paola Oliveri, Ochan Otim
Graduate Students: C. Titus Brown, Meredith L. Howard, Pei Yun Lee, Carolina Livi, Roger Revilla
Undergraduates: Sangeeta Bardhan, Christopher Franco, Albert Nguyen, Iman Perry[¶], Kirsten Welge, Yuan Xie, Kisha Young[§]
Research and Laboratory Staff: Research and Laboratory Staff: Carlzen Balagot, Barbara Barth, Ted Biondi, Chan Dinh, Ping Dong, Jessica Gamboa, Julie Hahn, Niñon Le, Patrick S. Leahy, Arnulfo Lorico, Jane Rigg, Deanna Thomas, Leah Vega, Lijun Wang, John Williams, Jane Wyllie, Jina Yun, Miki Yun
Consultants: Kevin Berney, Ramon Cendejas, Ian Lipsky
[*]*University of Hertfordshire, United Kingdom*
[†]*Institute for Systems Biology, Seattle, WA*
[‡]*Duke University, Durham, NC*
[¶]*Hampton University, VA*
[§]*Florida A&M University*

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Summary: The major focus of research in our laboratory is on gene networks that control development and their evolution. Our areas of research include the transcriptional mechanisms by which specification of embryonic blastomeres occurs early in development; structure/function relationships in developmental *cis*-regulatory systems; sea urchin genomics; and regulatory evolution in the bilaterians. Most of our work is carried out on sea urchin embryos, which provide key experimental advantages. Among these are an easy gene transfer technology; virtually unlimited availability of embryonic material, necessary for isolation of rare molecules such as transcription factors; an optically clear, easily handled embryo that is remarkably able to withstand micromanipulations; 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). At this point there is also an unusually rich collection of arrayed cDNA and genomic libraries for sea urchins, a fair amount of EST and genomic data and a very extensive repertoire of effective molecular technologies for experimentation on sea urchin gene regulatory systems. Sequencing of the *S. purpuratus* genome was recently approved by HGRI and will begin at the Baylor Sequencing Center in December, 2002. It is scheduled to take 10 months.

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 systems-level analysis of large regulatory networks. But one thing that has become apparent is that the only level of analysis from which explanations of major developmental phenomena directly emerge is the systems level represented by the regulatory gene network.

Current projects in the laboratory include the following: ***i. Analysis of the regulatory gene network underlying endomesodermal specification in S. purpuratus embryos.*** At present about 50 genes have been linked into this network. The architecture of the network is emerging from an interdisciplinary approach in which computational analysis is applied to data from gene expression knockouts, genomic sequence, and gene discovery projects, combined with experimental embryology. Regulatory and downstream genes required for skeletogenesis and for territorial specification have been isolated utilizing high-density arrayed cDNA libraries. A predictive model which indicates expected inputs and outputs of key *cis*-regulatory elements has been built and is being verified as the relevant *cis*-regulatory systems are found. Many direct experimental tests that can be carried out by altering gene expression in given embryonic cells can now be deduced from the architecture of the model. Many individual projects reported below are contributing to understanding of this network. ***ii. Analysis of cis-regulatory systems of territorially expressed genes*:** We are using *in vitro* mutagenesis and reconstruction of regulatory systems with synthetic DNA fragments in order to unravel the *cis*-regulatory "information processing system" by which genes determine where they are in the embryo, and whether to be expressed. Computational models are essential for interpretation and as guides to experimental analysis of these systems. Among genes expressed in the definitive territories and cell types of the embryo that are being studied in this way are: the *endo16* gene; the *wnt8* gene; the *brachyury* gene, *hbox12*, a gene encoding a homeodomain regulator of the very early skeletogenic lineage; *krox1*, a gene encoding a Zn finger transcription factor; *foxa*, a gene encoding a sea urchin HMF3β winged helix-type factor; *gatae*, an endodermal regulatory gene; *delta*, which encodes a Notch ligand; and *otx*, a transcriptional regulator which acts in many domains of the embryo, but which also plays a key role in endomesoderm specification. ***iii. Isolation, cloning, and functional analysis of transcription factors:*** We have developed technologies for purification of transcription factors from embryo nuclear extract, followed by microsequencing, and hence cloning. Transcription factors

that interact with target sites of known function can thus be characterized, and their embryonic provenance determined. Current research targets include several different transcription factors and a repressor that serves as a terminus of a signal pathway, all identified in studies on the *endo16* gene; and a transcriptional regulator of the *cyIIIa* aboral ectoderm-specific actin gene. ***iv. Sea urchin homeobox gene cluster:*** We have isolated the sea urchin *HOM-C* genes and shown that they reside in a single genomic cluster. Their genomic organization and pattern of expression during development are of particular comparative interest, since echinoderms (together with hemichordates) are the surviving sister group of all other major deuterostome members of the Animal Kingdom, i.e., chordates. We showed that whole cluster of *hox* genes is expressed in a colinear fashion in the rudiment of the adult body plan, and only two of the ten genes are used in the embryo at all. A current objective is to discover the downstream targets of the *hox11/13b* gene, which is expressed in the embryonic endoderm. ***v. Regulatory evolution:*** We are engaged in comparative assessments of *cis*-regulatory evolution in echinoderm species, comparing some key regulatory linkages in the endomesoderm gene network of a starfish to those discovered in the sea urchin. Starfish and sea urchins have evolved separately almost since the Cambrian. The endomesodermal gene network provides the opportunity of determining precisely which regulatory linkages have changed during evolution, and what are the consequences for the developmental process. ***vi. Computational approaches to regulatory gene network analysis:*** Regulatory gene networks for development cannot be informatively treated as equilibrium or steady-state systems. With Hamid Bolouri of the Institute for Systems Biology, Seattle, WA, a new approach to mathematical description of these developmental regulatory systems is being developed, with which to describe its unidirectional progression through successive spatial regulatory states. In addition several genome analysis tools are being development and a new project to build probability models of target sites for all known transcription factors in the endomesoderm network is under way.

Transcriptional Factor Research Resource Center (TFRRC)

The mission of the TFRRC of the Beckman Institute is to develop, extend and invent new technologies for working with transcription factors, particularly on a very small scale.

The innovative research that is carried out in the Center can be divided into two components: (*i*) affinity purification and microsequencing factors; (*ii*) computational models of the transcriptional regulatory processes and the development of the sea urchin genome database. The Research Center also shares responsibility for the Genomic Technology Facility. The Facility and the Center are an integrated unit in which advanced technologies are applied to the molecular biology of gene regulation. The main research material used in the Center is sea urchin embryos; these provide enormous technical advantages for transcription factor research, as reviewed elsewhere. Requests for collaborative research focused on transcription regulation are welcome: please contact Chiou-Hwa Yuh.

Purification, isolation, cloning of genes encoding transcription factors

Since its inception, a core objective of the TFRRC has been to improve methodology for direct isolation of transcription factors from DNA-protein complexes by affinity chromatography; to fractionate and partially purify the relevant proteins; and to determine enough of their structure by microsequencing to clone the mRNAs encoding them. This has never been easy to do, but it remains the only general approach by which a gene regulatory protein can be directly isolated. In the last year our capability to bring this technology to fruition has dramatically improved. In the last few months we have successfully obtained high quality protein sequence for the first time from an unprecedented five different previously unidentified transcription factors. These are an HNF6-class factor (with Dr. Ochan Otim); a CREB-class repressor; a gut-specific activator of the *endo16* gene; and an activator of an aboral ectoderm gene that had remained uncharacterized for about a decade (with Mr. C. Titus Brown.

Computational/experimental* cis-*regulatory analysis

Two major projects underway in the TFRRC. One, headed by Mr. Titus Brown, consists of an attempt to obtain the kinetic flow of regulatory inputs into the *cyIIIa* *cis*-regulatory systems, so that a complete model of this system can be built *in silico* rather like the *endo16* *cis*-regulatory system but with real-time inputs. The second, headed by C.-H. Yuh consists of a functional experimental analysis of *cis*-regulatory modules of the *otx* gene that were identified computationally by interspecific sequence comparison.

Computational aspects of endomesodermal gene network projects

With the addition of a Beowulf parallel computer stack to the Center, this is now the location of the major computational activities required for a large-scale gene network analysis in which we are involved, as a demonstration project. A computational scientist (Mr. Peter Clarke) is now in residence in the Center. A series of new computational research tools has emerged in the last year, developed under supervision of Hamid Bolouri: (*i*) the BioArray image processing package for quantitative and locational analysis of macroarray screens; (*ii*) a custom-built genomic DNA sequence annotation program called SUGAR (Sea Urchin Genome Annotation Resource); (*iii*) a new software package for trouble shooting and symmetry analysis of sets of shotgun genomic sequence reads (with Roy Britten and Mr. John Williams); (*iv*) statistical detection package for identification of *cis*-regulatory elements (this is still in the middle stages of development). In addition, a powerful new program, NetBuilder, is being adapted for creation of *cis*-regulatory logic models and networks. We also have available a newly-created program, FamilyRelations, built independently by Mr. Titus Brown for use in comparing large stretches of genomic sequence from related species.

The overall result is that the Center is developing powerful and novel capabilities for regulatory network genomics. These computational resources are all available, though a central server through which many different software applications that we have assembled can be run. Many Caltech labs and outside labs as well utilize this facility.

Technological repertoire of the Center

The Center is open to any outside scientists for whom its technological expertise is relevant, on a collaborative basis, to be arranged *ad hoc*. The current technologies and capabilities of the Center can be summarized as follows. These are capabilities that relate specifically to gene regulation and transcription factor isolation and analysis (though of course they might be useful for other problems as well).

Measurement of quantitative parameters of transcription factor/DNA interaction, by gel shift and capillary electrophoresis

Affinity chromatography, HPLC, microscale protein purification, and protein microsequencing

Miscellaneous technologies for cytological and biochemical characterization of transcription factor behavior and function

Design and execution of experimental *cis*-regulatory analysis

Computational analysis of DNA sequence similarity problems, array data reductions, and all aspects of gene regulatory network modeling

31. Analysis of new endomesoderm genes recovered by differential array hybridization

Andrew Ransick, Leah Vega

Analyses continue of several interesting transcription factors that are likely to play essential roles in regulating early endomesoderm specification. *Speve*, *Spgcm* and *Spfoxc* are all currently the subject of analyses to determine the complete mRNA expression profiles, determine what other genes in the Enodmesoderm Network the proteins interact with and what role they play in specification. Using morpholino antisense oligonucleotide microinjection in combination with quantitative PCR expression assays for endomesodermal marker genes and whole mount *in situ* hybridization, SpGcm expression has been found to be downstream of Delta/Notch signaling and upstream of pigment cells differentiation. These findings are entirely consistent with its expression profile, which initiates at 12 h in a subset of veg_2 lineage cells immediately adjacent to *Delta* expressing large micromere progeny. SpGcm is then expressed in the subset of the secondary mesenchyme precursors that give rise to the pigment cells. A comparison of the genomic sequence of *Spgcm* and *Lytechinus variegatus gcm* (*Lvgcm*) revealed the conserved non-coding DNA segments in the vicinity of the exons. Reporter constructs made with these conserved regions have given expression profiles resembling endogenous SpGcm mRNA, but to date have not produced an exact phenocopy of endogenous expression pattern. Additional neighboring DNA regions will be inserted into existing reporter constructs in an effort to identify all regulatory sequences. Similar analyses of *Speve* and *Spfocx* are being carried out, however to date only their mRNA expression profiles have been reported.

32. *brachyury* target genes in the early sea urchin embryo isolated by differential macroarray screening

Jonathan P. Rast, R. Andrew Cameron, Ping Dong

The transcription factor Brachyury plays a role in gastrulation and endoderm development throughout the Bilateria. Its expression is associated with cell shape changes and cell movement. We are continuing a project to characterize genes that are expressed downstream of *brachyury* during gastrulation of the sea urchin embryo as a means of understanding the mechanism of *brachyury* function. Screens were carried out with two different complex probes generated by subtractive hybridization on high-density arrays of embryonic cDNA libraries. Each of these probes is enriched for genes that are dependent on *brachyury* function for transcription. cDNA clones identified in these screens were characterized by sequencing. For interesting genes, the transcriptional activities in response to *brachyury* perturbation were further confirmed by real-time quantitative PCR analysis. Spatial domains of transcription were analyzed by *in situ* hybridization.

Genes isolated in this study fall into two major categories: genes expressed in the subset of the secondary mesenchyme cells (SMC) that will become pigment cells, and genes expressed in portions of the endoderm coincident with *brachyury* expression. The phase of *brachyury* expression that interests us is confined to the presumptive endodermal cells. The SMC genes are likely to be indirect targets of Brachyury-induced signaling from the surrounding endoderm to the central mesoderm, or the effects on these genes may be indirect consequences of gross vegetal plate disruption. The endodermal genes are candidates for direct transcriptional targets of Brachyury. Four of these genes are being studied in greater detail. Two of these genes, encoding a Calponin domain protein and a Gelsolin protein, are related to cytoskeletal modulators and may play a role in cell morphogenesis. This finding is consistent with the block in gastrulation induced by interfering with Brachyury function in sea urchins, and with known or suggested Brachyury function in other species. Two other endodermal target genes, a gene encoding an RNA binding protein and one encoding an organic cation transporter, are expressed in the archenteron and might be terminal differentiation products of the gut.

We are now comparing the sequence of BAC clones that contain these four downstream genes in order to define the *cis* connection through which *brachyury* operates, whether direct or indirect. We are working out strategies to expedite these network-scale *cis*-regulatory analyses. These methods include BAC modification using homologous recombination in bacteria and interspecies comparative methods to identify conserved *cis*-regulatory

elements. We are also expanding our analysis of the two differential screens to identify additional target genes. The results of this work in combination with other data suggest that *brachyury* transduces information about the state of endodermal specification to genes that modulate morphogenesis and genes that perform terminal functions in the gut.

33. Identification of secondary mesenchyme cell specific genes in sea urchin

Cristina Calestani

Sea urchin secondary mesenchyme cells (SMC) are the progenitors of pigment cells, blastocoelar cells, muscle cells and coelomic sac cells of the embryo. SMC are specified at the blastula stage from a vegetal embryonic territory, the vegetal plate. The central disc of cells of the vegetal plate is specified as SMC, while the ring of cells around it is specified as the endodermal progenitor cells. Notch signaling has been proven to be necessary for SMC specification. It has also been shown that LiCl treatment of embryos, which expands both presumptive endoderm and mesoderm territories, shifts the boundary of the Notch receptor localization toward the animal half. A more detailed understanding of the molecular and cellular mechanisms underlying mesoderm versus endoderm specification is a major goal.

In this work, a differential screening of a hatched blastula-stage macroarray cDNA library was performed using a comparison of mRNA populations from LiCl-treated embryos and dominant negative Notch (dnN) receptor-expressing embryos. The differential screening involved a subtractive hybridization between dnN RNA (driver) and LiCl-treated embryo single-strand cDNA (selectate), in order to lower the limit of detection on hybridized macroarray filters to genes expressed at about five copies per cell.

About 500 putative positive clones were identified and sequenced at the 5' end. About 30% of the clones had a significant homology (E-value < $1x\ 10^{-4}$) with genes in the NCBI DataBank. These genes encode enzymes, transcription factors, signal transduction proteins, extracellular matrix, membrane and cytoskeleton associated proteins.

Gene expression analysis was carried out on a subset of 65 clones with QPCR first and then with WMISH. About 60% of the clones analyzed by QPCR were positively regulated by N. They encode six different genes highly similar to: the transcription factor Glial Cells Missing (*gcm*); the polyketide synthase gene cluster (*pks*); three different members of the Flavin-containing monooxygenase gene family (*fmo*); and the Sulfotransferase 1 gene (*sult1*). In the hatched sea urchin blastula all these genes were expressed in the precursors of pigment cells and their expression was maintained in pigment cells throughout the gastrula and pluteus stages.

A functional analysis of one of the *fmo* genes was done using antisense technology and it was shown that *fmo* expression was essential for the biosynthesis of the sea urchin pigment echinochrome.

Further analysis of the other SMC candidate genes is in progress.

34. FoxA a key transcription factor in the endomesoderm gene regulatory network

Paola Oliveri, David R. McClay, Sangeeta Bardhan

SpFoxA is a forkhead domain transcription factor that is orthologous to the vertebrate *hnf3*-class genes. Its expression during the sea urchin development begins just after hatching and has two peaks: the first at the beginning of gastrulation (30 h), and the second at the time when the gut becomes subdivided (48 h).

SpfoxA transcripts are restricted to endoderm precursor cells and gut cells during all developmental stages. Around 30 hr of development a second domain of expression appears in the oral ectoderm corresponding to the future mouth. At the end of gastrulation (roughly 50 h) the levels of *foxA* gut expression are enhanced in fore- and hindgut.

A specific morpholino antisense oligo (MASO) has been synthesized to block *foxA* translation and thus its function. Embryos injected with α-FoxA show a clear block of gut development, obvious from the beginning of the gastrulation. An analysis of α-FoxA injected chimeric embryos, both in *Strongylocentotus purpuratus* and *Lytechinus variegatus*, shows that the *foxA* loss-of-function affects mainly veg_2 cells where the absence of this transcription factor directs them to a secondary mesenchyme cell (SMC) fate. QPCR experiments on the MASO injected embryos at different times of development show that FoxA plays a role mainly as a repressor in the early phases of expression (24-30 h), repressing *gcm*, *brachyury*, *foxB* and itself. On the contrary in the late embryo (48 h) FoxA positively acts on *endo16* and *gataE*. Thus *foxA* is a key regulator in endoderm specification, one of its functions is to repress one of the main pigment cells regulators, *gcm,* in cells that will become gut.

MASO-QPCR experiments linked *foxA* expression to the inputs received from *gataE*, the *wnt8/β-catenin* signaling system and *otx*, while *foxA* negatively regulates itself. In order to test the inputs predicted in the endomesoderm regulatory network, we decided to analyze the sequences surrounding the *foxA* gene for the ability in regulate the expression of a *gfp* reporter gene. We compared the *foxA* gene sequence between *S. purpuratus* and *L variegatus* (~50 My apart) using FamilyRelations. We identified 18 conserved regions outside the coding, in the analysis of a 50 kb fragment. Preliminary data on six of the fragments upstream of the *foxA* gene showed only two of them containing elements sufficient to direct the expression of *gfp*: a 1 kb fragment just upstream of the coding gives ubiquitous expression, and fragment around 20 kb upstream directs the expression of *gfp* in the oral ectoderm.

35. Pmar1 and the micromere regulatory gene network

Paola Oliveri

Micromeres and their immediate descendants have three known functions during sea urchins development: they are the source of an unidentified signal to the adjacent veg_2 cells that is required for normal endomesodermal specification; a few cleavages later they express Delta which triggers the conditional specification of the central

mesodermal domain of the vegetal plate; and they exclusively give rise to the skeletogenic mesenchyme of larva. We are studying the key components of the zygotic regulatory gene network that accounts for micromere specification and subsequent differentiation into skeletogenic cells. A central role is played by *pmar1* (paired-class micromere anti-repressor), encoding a paired class homeodomain transcription factor, which in micromeres acts as a repressor of a repressor. *pmar1* is expressed exclusively in micromeres only during the cleavage and early blastula stages. It starts its expression as soon as the micromeres are segregated, in response to Otx and β-catenin/Tcf inputs. The repressive nature of the interactions mediated by the *pmar1* gene product was shown by the identical effect of introducing mRNA encoding the Pmar1 factor, and mRNA encoding a Pmar1-Engrailed (En-Pmar1) repressor domain fusion. In both cases the effects are derepression of the *delta* gene and of skeletogenic genes normally expressed only in micromere descendants. These include the transcription factors *ets*, *tbr*, and *dri*, and a set of downstream skeletogenic differentiation genes, like *sm50*, *sm37*, *pm27*, *cyclophillin*, and *msp130*. The spatial phenotype of embryos injected with mRNA encoding Pmar1 factor or En-Pmar1 is expansion of the domains of expression of the downstream genes over most or all of the embryo. This results in transformation of much of the embryo into skeletogenic mesenchyme cells that express skeletogenic markers. The normal role of *pmar1* is to prevent, exclusively in the micromeres, the expression of an unknown repressor that is otherwise expressed throughout the embryo. This function accounts for the localization of *delta* transcription in micromeres, and thereby for the conditional specification of the vegetal plate mesoderm. It also explains why skeletogenic differentiation gene batteries normally function only in micromere descendants, and that the specificity of micromere function depends on global as well as on early localized inputs.

36. Placing SpKrL and SpSoxB1 in the endomesoderm network

*Carolina B. Livi, Lynne Angerer**

As part of a larger effort in the Davidson lab we are attempting to draw the network between all endomesodermal genes in the early development of the purple sea urchin. As a strategy to search for genes downstream of transcription factors, we have used morpholino anti-sense oligo nucleotides targeted to the transcriptional start site of their RNA message. This targeting prevents translation of the mRNA message in a sequence specific way. Previously, the effects of anti-sense morpholino oligos designed against SpKrl and SpSoxB1 in sea urchin embryos have been described (Howard *et al*., 2001 and Kenny *et al*., 2002). Some downstream target genes had been previously identified by semi-quantitative RT-PCR as well as by antibody staining analysis. In collaboration with the Angerer lab we checked the effects of blocking the translation of SpKrl as well as SpSoxB1 messages on the expression several genes from the endomesoderm network by real time quantitative PCR (RT QPCR). This way we would be able to identify some of their downstream target genes and place them within the existing network. It was previously known that SpKrl could lead to the reduction of SpSoxB1 protein levels. In this study we found no significant increase in SpSoxB1 mRNA levels in the absence of SpKrl, raising the possibility that SpKrl-mediated downregulation of SpSoxB1 protein levels is posttranscriptional. Several of the endomesodermal genes are affected when SpKrl activity is lost including SpEndo16, a commonly used endoderm marker in this system, and SpDpt. More recently others in the Davidson lab have found that the expression of several genes encoding regulatory proteins, SpGataE, SpHox11/13b and SpDelta to also depend on SpKrl for their expression (J. Rast and K. Young, unpublished results). Also, we have found that SpSoxB1 is capable of negatively autoregulating itself as its mRNA levels rise sharply in its absence. More work remains to be done to establish connections with other downstream target genes of these two factors.

**University of Rochester*

References

Howard, E.W., Newman, L.A., Olenksyn, D.W., Angerer, R.C. and Angerer, L.M. (2001) *Development* **128**:365-375.

Kenny, A.P., Oleksyn, D.W., Angerer, R.C. and Angerer, L.A. (2002) SoxB transcription factors are required for gastrulation in the sea urchin embryo. In preparation.

37. Function of a forkhead transcription factor *SpFoxB* in the sea urchin development

*Takuya Minokawa, César Arenas-Mena, Paola Oliveri, Christopher Franco, Brian Livingston**

Forkhead-class transcription factors are known for their role in the differentiation of endoderm and axial mesoderm in vertebrates. A forkhead transcription factor, *foxb* (formerly *Spfkh1*), was isolated by PCR screening from the sea urchin *Strongylocentrotus purpuratus* (Luke *et al*., 1997). According to the previous report (Luke *et al*., 1997), the spatial expression of *foxb* is limited to a part of the endoderm.

Recently, our systematic studies of gene regulatory networks in the early embryogenesis of sea urchin suggested that this gene should be of interest because of its complicated behavior in different perturbation experiments. This gene was clearly down-regulated when the function of early endomesodermal transcription factors (e.g., *gatae* and *krox1*) were inhibited. This suggested that f*oxb* is downstream of these endomesodermal genes. At the same time, however, this gene is drastically up-regulated when *pmar1* (a micromere lineage-specific homeodomain factor) expression was experimentally disrupted. This suggests that *foxb* is under the control of both endomesodermal genes and the micromere lineage-specific transcription factor *pmar1*. The spatial expression patterns of *SpPmar1* and the endomesodermal transcription factors do not overlap. So, the effect from either endomesodermal genes or *pmar1* must be indirect (via cell to cell interaction).

The purpose of this project is to understand the function of *foxb* in sea urchin embryogenesis and the regulation mechanisms responsible for *foxb* expression. Our recently improved whole mount *in situ* hybridization technology (Arenas-Mena *et al.*, 2000) revealed that *foxb* is

exclusively expressed in the PMCs (which are micromere descendants) at mesenchyme blastula stage, supporting the idea that this gene is down-stream of *pmar1*. Our quantitative PCR experiments indicated that the expression of *foxb* starts at 24 h when the PMC ingression into the blastocoel starts. These results suggest that the expression of *foxb* is correlated with PMC differentiation. Therefore, it is likely that the effect from endomesodermal transcription factors will be indirect through cell-cell interaction to the micromere lineage.

**University of South Florida*

References

Luke, N.H., Killian, C.E. and Livingston, B.T. (1997) *Develop. Growth Differ.* **39**:285-294.

Arenas-Mena, C., Cameron, A.R. and Davidson, E.H. (2000) *Development* **127**:4631-4643.

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

R. Andrew Cameron, Niñon Le, Jane Wylie, Paola Oliveri

Brachyury is a transcription factor involved in mesoderm specification in the sea urchin embryo as characterized in the endomesoderm specification studies being conducted in the Davidson laboratory. This gene is expressed in the oral ectoderm and the blastopore region of the archenteron. In order to determine the role of *cis*-regulatory sequence in the transcription of this gene, we adopted a new approach wherein random fragments of the genomic BAC clone were screened as GFP reporter genes. In the sequence from a BAC containing the *brachyury* gene we identified one large contig containing the gene and described the genomic structure of the *brachyury* transcription unit. This 22 kb stretch of DNA contains no other coding regions recognizable from Genbank. Using a 5' RACE strategy we place the start of transcription 370 base pairs 5' of the start of translation. There are seven different exons that lie over a 14 kb interval. The entire cDNA is only 3.7 kb in length and several of the introns are over 3 kb. Our functional fragment has been mapped to a 2 kb fragment that contains most of the intron between the 6th and 7th exons. Fragments of this region that contain consensus-binding sites for Gatae, Tcf and Foxa are positive in gel mobility assays. In other studies of the endomesoderm specification pathway, it has been shown that Foxa inhibits the expression of *brachyury*. A BAC containing a similar regio from the genome of *Lytechinus variegatus* has been sequenced. Using the FamilyRelations software developed by Titus Brown, we have identified conserved tracts of sequence in this intronic region. In addition, sequence tracts in other introns and at both ends of the transcription unit are conserved. Some of these support expression in GFP reporter constructs. The sum of the various elements necessary to define the expression of *brachyury* is coming into focus.

39. Functions of *Spdri* in the developing sea urchin I: Role in the PMCs and oral ectoderm specification networks

Gabriele Amore, Robert G. Yavrouian, Kevin J. Peterson***

Spdri is the sea urchin orthologue of the *Drosophila dead ringer* and mouse *bright* genes. It encodes a putative transcription factor belonging to the extended ARID (eARID) transcription factor family whose members are conserved in all metazoans. *Spdri* is a single copy gene. During sea urchin development it is activated first in the primary mesenchyme cells (PMCs) precursors from 14th cleavage until before these cells ingress the blastocoel; then in the oral ectoderm (OE) from the mesenchyme blastula stage on.

Spdri function was perturbed in two ways: by injecting zygotes with specific morpholino antisense oligonucleotides (*dri*-MASO) and by introducing *engrailed* fusion mRNA (*dri-en*). Both injections resulted in the inhibition of spiculogenesis (a late outcome of proper PMC differentiation) and in the abolition of OE specification. The injected embryos also failed to gastrulate.

The effects of these manipulations were further analyzed at a quantitative level by measuring variations in the transcription of a number of mesodermal, endodermal and ectodermal markers by use of real time quantitative PCR (QPCR). At a qualitative level changes in the mRNA pattern of expression of some of these genes were observed by whole mount *in situ* hybridization.

Spdri was shown to be necessary for the proper expression of PMCs differentiation markers such as *sm50, sm30, pm27, cyclofillin, ficolin* and *pm27*. Knock out of *Spdri*'s function did not interfere with the expression of genes that are required for the specification of these cells, such as *pmar1*, *ets* or *tbrain*. Nor was *Spdri* function required for the expression of *delta* so that secondary mesenchyme cells could differentiate in injected embryos, as assayed by the appearance of pigment cells and the proper expression of *gcm, fvmo, pks* and *decorin* (some SMC markers recently discovered in our laboratory).

During development, normal OE specification results in the expression and proper localization of genes such as *nk1, otp, gsc, nkx2.1* and *otx1/2*, and the confinement of *spec1* expression to the aboral side of the embryo. This is accomplished initially through *Spdri* independent mechanisms. However *Spdri* is required to maintain the expression of these marker genes so that if *Spdri* failed to be expressed, OE's specification was abolished. Interestingly *Spdri* was also found to be part of a reciprocal activation loop with *gsc*, so that after the onset of the expression of both genes in the OE, the expression of one supported that of the other. These results provided a first molecular-level description of the developmental commitment of cells to the OE fate. As we recorded the effects of the injections at several time intervals, we could follow the progression of events that see the regulatory function of *Spdri* in action and relate it with that of *gsc* and of all their target genes providing an initial understanding of the specification network of the OE.

**Drexel University College of Medicine*
***Dartmouth College*

40. Functions of *Spdri* in the developing sea urchin II: Role in vegetal specification and gastrulation

Gabriele Amore, David R. McClay, Andrew Ransick, Eric H Davidson

Because of the abolition of gastrulation observed upon injection of *dri*-MASO and *dri-en*, the role of *Spdri* in the specification of the vegetal plate was assayed. Neither the QPCR nor *in situ* analysis showed any effect of the perturbation on events that are relevant for the specification of this tissue. However when we measured Soxb1 localization, we discovered an anomalous distribution of this protein in the vegetal plate of the injected embryo. Normally this protein is cleared from this region and the absence of Soxb1 from the vegetal plate is required for the proper specification of this tissue. This is accomplished through signaling from the descendants of the micromeres (the PMCs precursors). As we discovered, the continuation of this signaling after 20 h requires *Spdri*. This was shown convincingly by use of chimeric embryos in which micromeres were switched between control and *dri*-MASO injected embryos. Only when *dri*-MASO was present in the micromeres the maintenance of Soxb1 clearance from the vegetal plate was impaired. Surprisingly, all of the chimeras were able to gastrulate. *Spdri* activation in the OE was found to be independent from PMCs *Spdri* function, therefore in the chimeras *Spdri* expression was maintained at least in one of its two normal sites. This implies that for gastrulation to occur *Spdri* expression must be maintained in either the PMCs or in the OE; only loss in both domains inhibits gastrulation.

Embryo microsurgery was also utilized to distinguish between autonomous and nonautonomous effects mediated by *Spdri* in the PMCs. In chimeras with *dri*-MASO injected PMCs, *Spdri* was found to be necessary for these cells to be able to interpret ectodermal cues and initiate spiculogenesis. Spiculogenesis was rescued later in these embryos by conversion of SMCs into spiculogenic mesenchyme. In the converse chimeras, expression of *Spdri* was required in the OE to provide some of the cues that are necessary for the growth of the skeleton inside the embryo. *sm30* transcription in the PMCs was dependent on both the PMCs and OE's presence of *Spdri,* pointing to the existence of both autonomous and nonautonomous mechanisms for the activation of this gene.

41. SpHnf6, a probable repressor during spiculogenesis

Ochan Otim

For the last few years, SpHnf6, a 52 kD *Strongylocentrotus purpuratus* transcription factor previously cloned in our lab, has presented us with a typical set of questions to answer regarding regulatory roles of a transcription factor in the development of *S. purpuratus* embryo. We are interested in determining when and where Hnf6 is active, its structure and function, and where Hnf6 fits in our dynamic model of the transcriptional network that underlies endomesodermal specification in *S. purpuratus*.

So far preliminary evidence we have accumulated relates *hnf6* to a limited number of genes. The evidence suggests that Hnf6 is both direct and indirect repressor of events leading to micromere specification in the developing embryo. For example, the levels of expression of terminal genes such as *sm27*, *cyclophillin*, *msp130*, *ficolin* and *sm50* which are exclusively expressed in the micromere descendent cells are upregulated in embryos injected with morpholino antisense oligonucleotide directed against Hnf6. Since injection of Hnf6-morpholino blocks the expression of Hnf6 protein, this implies a repressive effect on these terminal genes. We have also shown that Hnf6 interacts directly with the C-element of the *sm50* regulatory sequence in cooperation with other unidentified players. Efforts are underway using affinity purification technologies perfected in our Transcription Factor Research Resource Center to identify and characterize these unknown players.

At present, we are also screening for other high affinity DNA sequences to which Hnf6 binds. This is necessitated by observations that suggest that Hnf6 is likely using other regulatory sequences to control several genes. We are also looking for a possible splice variant of Hnf6 expressed during cleavage and early blastula stages.

Our laboratory is also interested in solving the endomesodermal regulatory gene network for the starfish, *Asterina Miniata* (see abstract by V. Hinman *et al*.). To that end, we have been able to isolate two isoforms of Hnf6 expressed in starfish: AmHnf6a and AmHnf6b. The two forms of Hnf6 differ only in the DNA sequences of the linkers between the Onecut and the Homeobox DNA binding domains. We are currently in the process of defining the structure/function of these two transcription factors.

42. *Spgatae*: Role in endomesoderm specification and *cis*-regulatory analysis

Pei Yun Lee

Spgatae is the *S purpuratus* ortholog to vertebrate Gata genes *4/5/6*. The expression of *Spgatae* is first detected in the presumptive secondary mesenchyme cells (SMCs) during the hatching blastula stage. Its expression expands to include both the future SMCs and the endoderm at the beginning of gastrulation. In the gastrula, *Spgatae* is expressed in the foregut and hindgut. By the end of embryogenesis, *Spgatae* expression in the hindgut has expanded to include part of the midgut as well. *Spgatae* expression is also detected in the pigment cells and coelomic pouches.

Quantitative PCR on embryos whose *Spgatae* expression was knocked down reveals that *Spgatae* activates many endoderm and mesoderm transcription factors. Combined with data from similar perturbation experiments on other genes, the data suggest that a role of *Spgatae* is to lock in the commitment of the endomesoderm developmental program.

An analysis of the *cis*-regulatory region of *Spgatae* will verify the connections between *Spgatae* and other genes in the endomesoderm network at the DNA level and further refine the network model. FamilyRelations has been shown to be an effective tool in identifying potential *cis*-regulatory elements (Yuh *et al.,*

2002). Comparison of the *S. purpuratus* and *L. variegatus gatae* genomic regions using FamilyRelations identified 28 conserved regions. Eleven of these regions have been cloned into GFP reporters and tested by injection into sea urchin eggs and observation of GFP expression. Of these, a 600 bp fragment in intron 1 so far is the major *cis*-regulatory element responsible for *Spgatae* expression in the vegetal plate at the hatching and mesenchyme blastula stages. This fragment is being studied in greater detail and more fragments are being cloned and tested.

43. *cis*-Regulatory study on the sea urchin *otx* locus using comparative sequence analysis

Chiou-Hwa Yuh

The *otx* (orthodenticle-related) gene in sea urchin plays an important role in early embryogenesis. SpOtx exists in two forms (and E) that are generated by alternative RNA splicing from a single *Spotx* gene. Three distinct SpOtx(E) and one SpOtx( ) mRNA appear to be controlled by different regulatory elements as indicated by the different expression pattern obtained with *in situ* hybridization from a previous study. -otx transcripts are first ubiquitous from maternal messages, then accumulate zygotically in the vegetal plate endomesoderm. We could not see oral ectoderm expression at 24 h. After 24 h, -otx transcripts decline sharply in prevalence. E1/2 Otx transcripts are localized to the oral ectoderm during the late blastula to early gastrula stage and are strongly expressed in the vegetal plate endomesoderm as well. In the late embryo, expression is in the hood region of the oral ectoderm and gut. The E3 otx transcription unit begins to be expressed ubiquitously, during cleavage, and is still being expressed ubiquitously at mid-blastula stage. As gastrulation begins, expression is seen clearly in the invaginating archenteron, and in the ectoderm on one side. OtxE3 transcription ceases soon after this stage.

To understand the regulatory mechanism, the BAC clones containing the *otx* gene have been isolated from two different species of sea urchin. Using the newly developed program "FamilyRelations," we were able to find seventeen partially conserved regions, most a few hundred base pairs long. These were amplified from the *S. purpuratus* BAC DNA by PCR, inserted in an expression vector driving a CAT reporter, and tested for *cis*-regulatory activity by injection into fertilized *S. purpuratus* eggs. The regulatory activity of these constructs was assessed by whole mount *in situ* hybridization (WMISH) using a probe against CAT mRNA. Of the 17 constructs, 11 constructs displayed spatially restricted regulatory activity, and six were inactive in this test. The domains in which the *cis*-regulatory constructs were expressed are approximately coincident with results from a WMISH study on *otx* expression in the embryo. Four separate *cis*-regulatory elements that specifically produce endomesodermal expression were identified (11-CAT, 14-CAT, 15-CAT and 17-CAT). Among them, three elements are present in front of OtxE1/2 transcription start site, and 17-CAT is located immediately upstream of Otx . According to QPCR results, OtxE1/2 transcripts seem to be effected by GATA-morpholino, cadherin mRNA and engrailed-Otx. But, the Otx transcript does not seem to be affected. To understand these functional perturbations on the *cis*-regulatory system of the *otx* gene, we employed co-injection experiments. Otx11, 14 and 15 elements are responsible for these inputs, and consistently, there are 4, 6 and 3 GATA sites on 11-CAT, 14-CAT and 15-CAT, respectively. There are one, one and three Tcf sites on 11-CAT, 14-CAT and 15-CAT, respectively. The number of Otx binding sites does not correlate with the effect. On the other hand, the Otx transcript does not seem to be affected, and the corresponding Otx17 element is not responsive to those perturbations.

Further experiments on mutation of those binding sites and fine mapping of the entire elements are ongoing. The ultimate aim is to identify the target site sequences of the *cis*-regulatory elements of the *otx* gene and the interrelationships of these genes constitute the physical basis of the network architecture.

44. Regulation and functions of a posterior group Hox gene

César Arenas-Mena, R. Andrew Cameron, Niñon Le

The Hox gene cluster is involved in axial patterning processes and is conserved throughout the bilaterian animals. Of the ten genes in the single sea urchin Hox cluster only two are expressed during embryogenesis, but neither have any axial patterning role. However, the five most posterior genes of the cluster are expressed during post-embryonic stages in a colinear fashion in the posterior coelomic compartments of the bilateral larva, where they may play a role in the development of the adult body plan.

We have used an evolutionary comparative genomic approach in order to better define the non-coding transcriptional control regions of the most posterior Hox gene (SpHox11/13b). The non-coding conserved genomic fragments have been subcloned into a GFP reporter construct and are currently under study. Previous results indicated that the transcription factors Krox, Otx and the β-catenin/Tcf signaling pathway positively regulate SpHox11/13b. Thus these regulatory inputs are expected to hve their corresponding *cis*-regulatory sequences, i.e., transcription factor binding sites, in the transcriptional regulatory DNA of SpHox11/13b. Perturbation experiments will be undertaken to test this expectation.

SpHox11/13b is expressed dynamically in the presumptive endoderm during late cleavage and gastrulation; by the end of embryogenesis it is restricted to the hindgut and anus where it continues to be expressed in larval stages. Morpholino knockdown experiments suggest that SpHox11/13b regulates some cell adhesion functions during gastrulation. In addition, decreasing SpHox11/13b expression reveals a repressive function in the hindgut for late midgut-specific genes such as Endo16. This is probably mediated by the transcription factor Gatae, which is also repressed by Hox11/13b.

The expression of the most posterior Hox gene in the hindgut and anus is of evolutionary interest, because it is conserved among all animals studied so far. This may represent an ancestral function in the posterior digestive tract. It is important to know how this expression domain

is established in a bilaterian larva, which is considered to be at the ancestral level of organization for bilaterians.

45. Regulatory gene network evolution: A comparison of endomesoderm specification in starfish and sea urchins

Veronica F. Hinman, Albert Nguyen, Kirsten Welge

We are undertaking an evolutionary comparison of the regulatory network of transcription factors underlying the specification of endomesoderm in sea urchins and starfish. The recent extensive analysis of this network in sea urchins has provided a unique opportunity for a comparative investigation to elucidate mechanism of evolution at this level. We would like to answer questions such as, which components of such a regulatory system are conserved, how are changes incorporated into a network, and importantly, how do these changes relate to the evolution of morphology? The starfish *Asterina miniata* has been developed as an ideal experimental model for this analysis. Gametes are readily available and gene transfer and perturbation of gene products have been performed. Starfish last shared a common ancestor with sea urchins around 500 million years ago. While many aspects of their early embryonic development are conserved, there are some key differences that will provide for meaningful evolutionary comparisons of their underlying developmental processes.

We have isolated starfish cDNA orthologues to some of the transcription factors known to be part of the specification network in sea urchins (i.e., *otx*, *krox*, *gatae*, *foxa*, *brachyury*, *tbrain*, and *foxb*). We have analyzed and compared their temporal and spatial expression and their epistatic interactions using various forms of perturbation. This has allowed us to construct a regulatory network of these genes in the starfish. This analysis has shown that many regulatory connections are conserved between these two taxa, including some early interactions that are thought to initiate and stabilize the endoderm specification network. Among the differences, we have noted that Tbrain is incorporated into the endoderm-specification network in starfish while it is involved in primary mesenchyme cell specification in sea urchins. This is reflected in the difference in spatial expression of this gene in the two animals. We can now test whether this difference also accounts for changes in differentiation products and hence the morphological evolution of the cell lineages between sea urchins and starfish.

46. An enhancer trap strategy

R. Andrew Cameron, Paola Oliveri, Niñon Le, Jane Wyllie

We are analyzing two clones obtained during our random scan of the *Strongylocentrotus purpuratus* genome for sequences that support expression of a reporter gene in embryos. Originally, reporter gene constructs containing a green fluorescent protein (GFP) coding region were injected into sea urchin zygotes and observed for expression. We detected expression of GFP in 14 of the 108 individual constructs tested. Some of these constructs produced tissue specific expression and others were ubiquitous. The temporal expression patterns were also diverse.

We chose two fragments that confer spatially restricted expression for further analysis: C20, a pigment cell-specific fragment and 2D12, a gut-specific fragment. The sequences are not unusual; they have a GC content of 35%; about the average for sea urchin genomic DNA. Both sequences identified small 15-16 base pair matches to the purple sea urchin repeat collection at a low significance, e.g., these matches are expected to exist in a random sequence of the size examined between 44 and 690 times out of 1000. Comparison to the Genbank non-redundant DNA database by BLASTX and BLASTN revealed only one significant match at 1e-06 for the C20 sequence. The match was to a reverse transcriptase-like sequence.

To more narrowly define the functional elements in these genomic sequences we subcloned fragments for further gene transfer analysis. Subfragments of the C20 clone were made by deletions from internal restriction sites to give three segments across the length of the original sequence. The 5' 1777 base pairs in themselves did not support any expression. This leaves about 1.2 kb at the 3' end of the original 2.9 kb sequence that provides the temporal and spatial control by which the clone was selected. Of the transcription factors known to be involved in endomesoderm specification, Gatae, Foxa and Tcf have well characterized binding sites. A search for these sites revealed several in the C20 subfragment. Although the 2D12 fragments have not all been analyzed, one 2 kb fragment derived by deleting about 1 kb from near the middle recapitulates the control achieved by the whole segment. Further deletion of 1.5 kb from the 3' end results in loss of spatial control. The last experiment suggests that the 3' 1.5 kb contains a spatial repressor and the 5' region contains a general enhancer.

47. High-density arrayed library technology at the Sea Urchin Genome Resource

Julie Hahn, Ted Biondi, Arnulfo Lorico, Ping Dong, Jina Yun, Jonathan P. Rast, R. Andrew Cameron

High-density arrayed libraries and the automated technology associated with them greatly facilitate the identification of individual genes, differential screening strategies, the study of genomes and many other aspects of molecular developmental biology. Our automated facility, centered around a colony arraying and spotting robot, has been used in support of several library preparation and sequencing efforts that together constitute a Sea Urchin Genome Resource. The collection of materials in the Resource now includes 14 cDNA libraries and six genomic libraries. Descriptions of these materials are available on the Resource web site (http://sugp.caltech.edu/). For an idea of the scale of the Facility's operation, we arrayed more than 2 million clones and produced over 700 high-density filters ready for screening in three years of operation.

The sea urchin community has made numerous requests for library filters which have resulted in continuing requests for clones since each filter set can be screened many times. Since libraries requested in previous

years may result in clone requests this year we document 17 laboratories requesting clones and filters in the past two years. Outside of our own laboratory, about 500 clones from the arrayed libraries were prepared as glycerol stocks and distributed to the users. In addition, five users have requested libraries but not requested clones. This level of response indicates that the Sea Urchin Genome Resource continues to be useful to the greater biological community.

Furthermore, the echinoderm libraries that the Facility has produced have formed the essential starting materials for the genomics of this model system. The *S. purpuratus* BAC library arrayed in the Facility has already been used for both an ~80,000 BAC-end sequence project, and the gene network project; an offshoot of which has been the acquisition of about 6% of the total genomic sequence (the genome is 800 mb). The National Human Genome Research Institute has designated the sea urchin as a high priority for genome sequencing (http://www.nhgri.nih.gov/NEWS/sequencing.html) and Baylor College of Medicine, Human Genome Sequencing Center will begin full-scale sequencing in December, 2002. We have transferred the BAC library to the Baylor HGSC in preparation for the sequencing effort.

48. Precambrian animal life: Putative developmental and adult cnidarian forms from S. W. China

Paola Oliveri

The evolutionary divergence of cnidarian and bilaterian lineages from their remote common ancestor occurred at an unknown time before the Cambrian, since crown group representatives of each are found in Lower Cambrian fossil assemblages. Analysis of thousands of thin sections of rock under bright field and polarized light lead to the identification of a variety of putative embryonic, larval and adult microfossils deriving from Precambrian phosphorite deposits of S. W. China, which may predate the Cambrian radiation by 25-45 million years. We already reported the identification of microfossils of possible bilaterian origin (Chen *et al*., 2000). Recently we focused our attention on forms that are most probably of cnidarian affinity. Large numbers of fossilized early planula-like larvae were observed. Though several forms are represented, the majority displayed remarkable conformity, which is inconsistent with the alternative that they are mineral inclusions of non-biological origin. Some of these fossils are preserved in such high resolution that individual cells can be discerned. We confirm in detail an earlier report of the presence in the same deposits of tabulates, an extinct crown group anthozoan form. Other sections reveal structures that most closely resemble basal modern corals. A large number of fossils similar to modern hydrozoan gastrulae were also observed. These again displayed great morphological consistency. Though only a single example is available, a microscopic animal remarkably similar to a modern adult hydrozoan is also identified. These new observations indicate the existence of a diverse and already differentiated cnidarian fauna, long before the Cambrian evolutionary event. It follows that at least stem group bilaterians must also have been present at this time, consistent with previously reported observations (Chen *et al*., 2000) and the most conservative calculation of the molecular divergence that indicates the latest bilaterian common ancestor could not have existed less than 600 million years ago.

Reference

Chen, J.Y., Oliveri, P., Li, C.W., Zhou, G.Q., Gao, F., Hagadorn, J.W., Peterson, K.J. and Davidson, E.H. (2000) *Proc. Natl. Acad. Sci USA* **97**:4457-4462.

49. Database software for the Sea Urchin Genome Project web site

Titus Brown, Ian Lipsky, Ramon Cendejas, Kevin Berney, R. Andrew Cameron

The Sea Urchin Genome Project web site (http://sugp.caltech.edu/) is the distribution center for sequence and annotation information related to the sea urchin genome and the macroarray libraries of the Sea Urchin Genome Resource. The Database section of the web site includes web pages for searching the sequence resources accumulated over the past several years. The sequence data as well as other information is linked to the plate and well location for each of the library clones in the various libraries that make up the resource. The sequence information is mostly in Genbank but it represents an easily searched small database here. There are over 100,000 sequences in our sequence database. The machine base for the web site is now two Dell Poweredge 1300 machines, each with two processors and two disks. A third Pentium machine is used for annotation runs and the development of Cartwheel (see the separate report). In addition, we have a 17-node Beowulf cluster that is fully integrated into the system so that it can be used for larger computational jobs in a distributed fashion. The software that supports the web site has been completely revamped to make it more flexible and reliable. The new version is purposely more extensible as well.

In addition to our collection of sequence data, users can search our data using filter spot locations and retrieve sequence information, probes that hybridized to the spot pair, sequence derived from an individual clone and the names of genes that match the derived sequence. To make the filters more useful we have conducted several "junk" screens to identify prevalent, low information-containing clones and publish them in the database. Combining the EST data, cDNAs identified in the various complex screens mentioned above and the junk screens renders the identification of false positives from screening trials much less probable.

50. Understanding gene regulation through motif analysis

Meredith L. Howard

The developmental program directing the growth of the sea urchin from a single-cell fertilized egg to an embryo is encoded in the organism's genomic DNA. The essence of this program is a network of genes encoding transcription factors and the *cis*-regulatory modules controlling the expression of those genes. Each module can receive multiple inputs at various sequence-specific target sites for other transcription factors in the network, and these signals are integrated into a single output

resulting in the gene being turned on or off in different areas of the developing organism at different points in time. Understanding the developmental process therefore requires finding the functional linkages of the network – connecting the output of regulatory genes to the genomic target sites to which those products bind to activate further rounds of specification. This task is made challenging by our incomplete understanding of how transcription factors discern functional target sites from the vast population of non-functional sites with the same sequence in the genome.

In order to study how functional target sites within *cis*-regulatory modules differ from non-functional sites scattered throughout the genome, we are assembling a database of known functional target sites and consensus sequences of transcription factors known to be active in the early development of the sea urchin. The database will be used to map all target sites occurring within the intergenic regions of genes being studies in our lab. The *cis*-regulatory modules of many of these genes are either already partly understood or in the process of being dissected. Computational methods will be used to study differences in flanking sequences, relative positioning and orientation, and other characteristics of functional and non-functional sites.

In a related effort, an algorithm has been written to search for as yet unidentified motifs involved in activating or repressing co-regulated genes. In this way, we may be able to fill in gaps in the endomesodermal gene network. The program takes as input multiple sequences and searches for motifs of user-determined size that are common between all sequences. Ideally, each input sequence is a concatenation of patches identified by seqcomp/FamilyRelations as being conserved non-coding regions upstream or intronic to each gene of interest. It is hoped that the potential of this approach will be realized as the sequencing of the sea urchin genome proceeds.

51. Development of an analysis server for sequences

C. Titus Brown, Tristan De Buysscher, Meredith L. Howard, Barbara J. Wold, R. Andrew Cameron

Genomics experiments depend to a large extent on correlating, comparing, and visualizing different analyses of genomic data. The Cartwheel Project (http://cartwheel.caltech.edu/) is an open-source project that provides a server-based solution to this problem. The Cartwheel Web server enables biologists to analyze genomic data in several ways and is currently in use by about 15 labs; the framework underlying the server is continually being expanded to provide new interfaces and utilities for dealing with sequence data.

Cartwheel is being developed in conjunction with our comparative sequence analysis viewer.

52. Continuing development of a two- and three-way comparative sequence analysis viewer

C. Titus Brown, Ramon Cendejas, Tristan De Buysscher, Barbara J. Wold, R. Andrew Cameron

We have continued development of The FamilyRelations package for visualization of comparative sequence analyses, extending it from display of two-way comparisons to also display three-way comparisons. We have also incorporated a single-sequence annotation view to display correlations between a variety of BLAST analyses and gene finding programs.

Publications

Bolouri, H. and Davidson, E.H. (2002) Modeling DNA sequence-based *cis*-regulatory gene networks. *Dev. Biol*. **246**:2-13.

Bolouri, H. and Davidson, E.H. (2002) Modeling transcriptional regulatory networks. BioEssays. In press.

Brown, C.T., Rust, A.G., Clarke, P.J.C., Pan, Z., Schilstra, M.J., De Buysscher, T., Griffin, G., Wold, B.J., Cameron, R.A., Davidson, E.H. and Bolouri, H. (2002) New computational approaches for analysis of *cis*-regulatory networks. *Dev. Biol*. **246**:86-102.

Cameron, R.A., Oliveri, P., Wyllie, J. and Davidson, E.H. (2002) *cis*-Regulatory activity of randomly chosen genomic fragments from the sea urchin. In preparation.

Chen, J.Y., Oliveri, P., Gao, F., Dornbos, S.Q., Li, C.W., Bottjer, D.J. and Davidson, E.H. (2002) Precambrian animal life: Probable developmental and adult cnidarian forms from Southwest China. *Dev. Biol.* **248**:182-196.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.-H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P., Revilla, R., Rust, A.G., Pan, Z.J., Schilstra, M.J. Clarke, P.J.C., Arnone, M.I., Rowen, L., Cameron, R.A., McClay, D.R., Hood, L. and Bolouri, H. (2002) A genomic regulatory network for development. *Science* **295**:1669-1678.

Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.-H., Minokawa, T., Amore, G., Hinman, V., Arenas-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P., Revilla, R., Schilstra, M.J. Clarke, P.J.C., Rust, A.G., Pan, Z.J., Arnone, M.I., Rowen, L., Cameron, R.A., McClay, D.R., Hood, L. and Bolouri, H. (2002) A provisional regulatory gene network for specification of endomesoderm in the sea urchin embryo. *Dev. Biol*. **246**:162-190.

Erwin, D.H. and Davidson, E.H. (2002) The last common bilaterian ancestor. *Development* **129**:3021-3032.

Oliveri, P., Carrick, D.M. and Davidson, E.H. (2002) A regulatory gene network that direct micromere specification in the sea urchin embryo. *Dev. Biol*. **246**:209-228.

Ransick, A., Rast, J.P., Minokawa, T., Calestani, C. and Davidson, E.H. (2002) New early zygotic regulators of endomesoderm specification in sea urchin embryos discovered by differential array hybridization. *Dev. Biol*. **246**:132-147.

Rast, J.P., Cameron, R.A., Poustka, A.J. and Davidson, E.H. (2002) *brachyury* target genes in the early sea urchin embryo isolated by differential macroarray screening. *Dev. Biol*. **246**:191-208.

Yuh, C.-H., Brown, C.T., Livi, C.B., Rowen, L., Clarke, P.J.C. and Davidson, E.H. (2002) Patchy interspecific sequence similarities efficiently identify positive *cis*-regulatory elements in the sea urchin. *Dev. Biol*. **246**:148-161.

Anna L. Rosen Professor: Scott E. Fraser
Member of the Beckman Institute: Russell E. Jacobs, Jerry E. Solomon
Senior Research Associate: Thomas J. Meade, Carol W. Readhead
Member of the Professional Staff: Gary R. Belford
Visiting Associates: Eric T. Ahrens, James F. Amatruda, Peng-Sheng Chen, Adam N. Mamelak, Neil Segil, Peter H. Siegel, John Charles Wood
Visitors: Andres Collazo, Kalpana Desai, Rex A. Moats, John B. Wallingford, Ying You
Senior Research Fellows: Rusty Lansford, Angelique Louie
Senior Postdoctoral Scholar: Paul Kulesa
Postdoctoral Scholars: Axel Blau, Thomas DeMarse, Mary Dickinson, Ivan Dmochowski, Frank Femia, Reinhard Köester, David Koos, Melanie Martin, Helen McBride, Cyrus Papan, Robia Pautler, Seth W. Ruffins
Graduate Students: Matthew Allen, Magdalena Bak, Joseph Duimstra, Andrew Ewald, Ying Gong, Anita Gould, Brian Johnson, Elizabeth Jones, Jason Kastner, Elizabeth Krider, Carole Lu, Jeremiah Miller, Carlo Quinonez, Daniel Wagenaar
Research and Laboratory Staff: Carolyn Allen, John Carpenter, Sonja Collazo, Daniel Darcy, Tatyana Demyanenko, Mary Flowers, Tim Hiltner, Aura Keeter, David Kremers, Ha Yong Edward Lim, Edriss Merchant, Jesse Munoz, Palliakaranai T. Narasimhan, John-Carl Olsen, Agavni Petrosyan, Alan W. Pinkney, Steven M. Potter, Miriam Scadeng, Sambasivam Sendhil Velan, Yuling Sheng, Jeffrey O. Smith, Lauren Somma, Stephen Speicher, Diana St. James, Kannika Sypanyo, Julian Michael Tyszka, Christopher Waters, Yan Wu, Changjun Yu, Joseph Zadeh
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Summary: Biological imaging offers one of the most direct links between the wealth of data made available by modern molecular biology and the rapidly growing fields of developmental and cell biology. Imaging permits direct tests of the molecular and cellular interactions proposed from *in vitro* experiments. Our goal is to perform test experiments in the intact system so that we can advance from what might happen to what does happen in the natural biological context. Thus, our work must overcome two significant challenges: visualizing cells and structures in the intact organism; reliably and indelibly marking cells so they can be followed without perturbing their development or behavior. An additional challenge is to interpret the findings, requiring computational tools to visualize and interact with the data, as well as to simulate the molecular networks operating within and between cells.

Recent progress in the laboratory has addressed each of our challenges. New multispectral laser scanning microscopy techniques have been developed that permit multiply labeled cells to be followed without cross talk between the signals. Improved tools for TPLSM have made the most challenging microscopy techniques accessible to non-specialists. Dramatically improved spatial and temporal resolution of our magnetic resonance microscopy allows even rapidly moving structures such as the developing heart to be analyzed. Atlas construction and volume rendering efforts allow the multidimensional data sets to be shared with and used by others. Finally, new approaches for stochastic simulations have allowed this powerful means for analyzing the networks of interacting molecules inside of cells to be applied to multi-cellular assemblages. Together, these improvements open new opportunities for bringing our experimental analysis to intact systems and make it more straightforward to interact with the resulting data.

53. Diffusion tensor imaging of the mouse brain

J. Michael Tyszka, Robia G. Pautler, Russell E. Jacobs

Magnetic resonance diffusion tensor imaging (DTI) is now well established for neuroanatomical studies of water diffusion anisotropy in both normal and pathological human brain. The tensor measurement is generally calculated on a voxel-by-voxel basis from seven or more diffusion-weighted MR images in which the direction and magnitude of the field gradient induced diffusion sensitivity is varied. The method allows indirect measurement of fiber orientation in white matter tracts from the principal eigenvector corresponding to the largest eigenvalue of the resulting tensor. Fewer DTI studies have been performed in mice (1,2), but this too shows great promise for phenotyping neuroanatomy in mutant and knockout strains. DTI studies in specially fixed mouse brains were performed using the 12 Tesla Bruker Avance

imaging system in the Biological Imaging Center. An optimized diffusion-weighted UFLARE (3) acquisition pulse sequence was used to acquire true three-dimensional imaging data with an isotropic spatial resolution of 117mm. The diffusion tensor was calculated from seven 3D diffusion weighted images. Auxiliary images such as anisotropy indices and eigenvectors were calculated from the tensor field. White matter tracts could be traced from the principal eigenvector field by solving the Frenet equation using an adapted ODE solver (4). Future collaborative studies using this newly implemented technique will characterize white matter pathology in mouse models of neurological diseases such as multiple sclerosis (with Carol Readhead and Melanie Martin), Alzheimer's disease, and Down's syndrome.

References

(1) Zhang, J.Y., van Zijl, P.C.M. and Mori, S. (2002) *Neuroimage* **15**:892-901.

(2) Mori, S., Itoh, R., Zhang, J.Y., Kaufmann, W.E., van Zijl, P.C.M., Solaiyappan, M. and Yarowsky, P. (2001) *Magn. Reson. Med.* **46**:18-23.

(3) Norris, D.G. (1991) *Magn. Reson. Med.* **17**:539-542.

(4) Basser, P.J., Pajevic, S., Pierpaoli, C., Duda, J. and Aldroubi, A. (2000) *Magn. Reson. Med.* **44**:625-632.

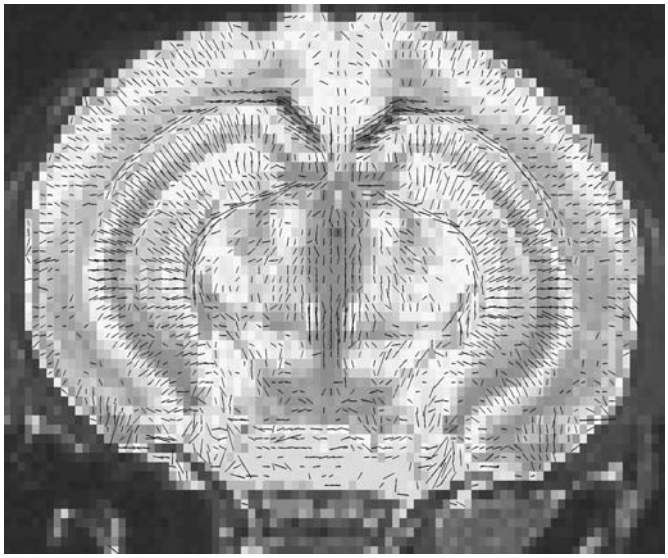


Figure 1. In-plane components of the principal DTI eigenvector overlayed in black on the corresponding isotropic diffusion weighted section through the hippocampus of a fixed C57/BL6 mouse brain

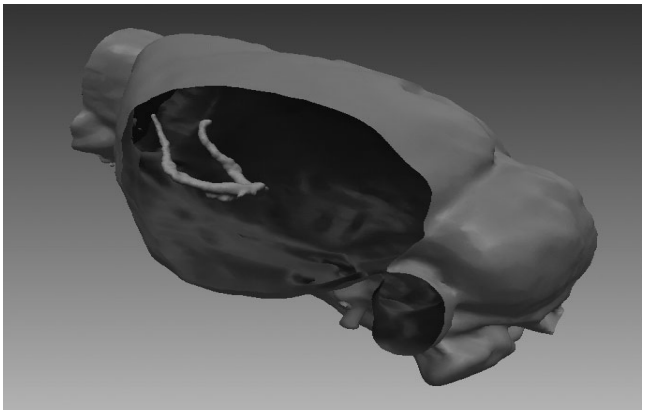


Figure 2. DTI tractography of a fixed C57/BL6 mouse brain. The tract shown in red (left center) resulted from several hundred subtracts originating from around a seed point in the anterior commisure.

54. Magnetic resonance microscopy and spectroscopy of Japanese quail embryos *in ovo*

J. Michael Tyszka, Robia G. Pautler, Rusty Lansford, Russell E. Jacobs

The Japanese quail embryo provides an excellent model for developmental biology with clinical applications and is an ideal system in which to combine dynamic imaging and molecular genetics technologies. Quail reach sexual maturity quickly and are prolific breeders. The avian embryo is extremely hardy, permits unsurpassed accessibility for imaging and tissue manipulation, and has long been a favorite of classical embryologists. The quail embryo has been used in studies involving limb regeneration, neural transplantation, blood vessel development, cardiac studies as well as a multitude of developmental diseases such as Fetal Alcohol Syndrome and metabolic disorders. Beyond day 5, the embryo becomes increasingly difficult to image *in ovo* using conventional optical microscopy, providing a valuable application for magnetic resonance microscopy, which is not limited by optical penetration. However, as the embryo develops, gross involuntary motion becomes more frequent, and the need for rapid data acquisition increases [Figure 1]. Echo-planar MR sequences provide rapid data acquisition, but are sensitive to susceptibility gradients within a sample, particularly at high field. Methods such as RARE (1) and UFLARE (2) sacrifice acquisition speed for robustness to B_0 inhomogeneities.

Quail embryos (stages E5-E10), were imaged using a 12T Bruker Avance DRX imaging system [Figure 2]. All experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee. Heavily T_2-weighted MR images of living quail embryos were acquired with isotropic in-plane spatial resolutions between 270μm and 540μm. Typical echo-train durations of between 200ms and 400ms were achieved, which renders the embryo stationary in the image, even during periods of gross motion. Single voxel magnetic resonance ^{1}H spectra can also be acquired from the brains of anesthetized quail embryos, allowing semi-

quantitative measurement of intracellular creatine, choline and *myo*inositol **[Figure 3]**.

Magnetic resonance imaging and spectroscopy are capable of providing both morphological and biochemical information from the developing quail embryo non-invasively at developmental stages beyond E5, where conventional optical microscopy becomes penetration limited.

References

(1) Hennig, J., Nauerth, A., Friedburg, H. (1986) *Magn. Reson. Med.* **3**:823-833.
(2) Norris, D.G. (1991) *Magn. Reson. Med*. **17**:539-542.

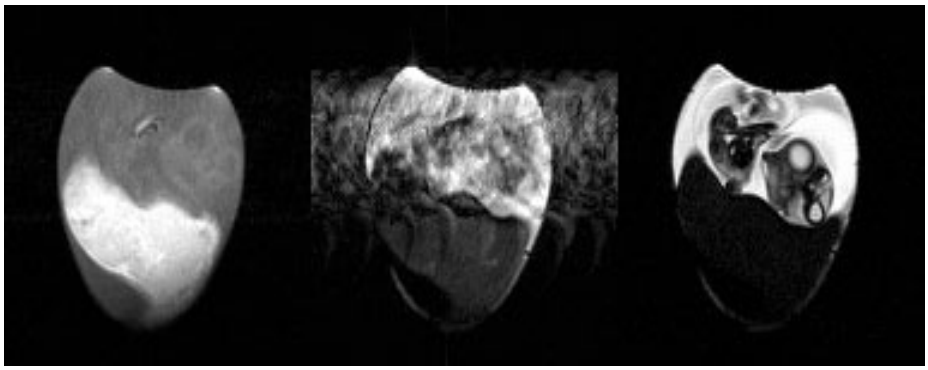


Figure 1: Comparison of conventional T_1-weighted spin echo (left), conventional multi-shot RARE (middle) and single-shot RARE (right) imaging of the un-anesthetized quail embryo. T_1-weighted imaging shows almost no tissue contrast, and multi-shot RARE is severely compromised by motion artifacts.

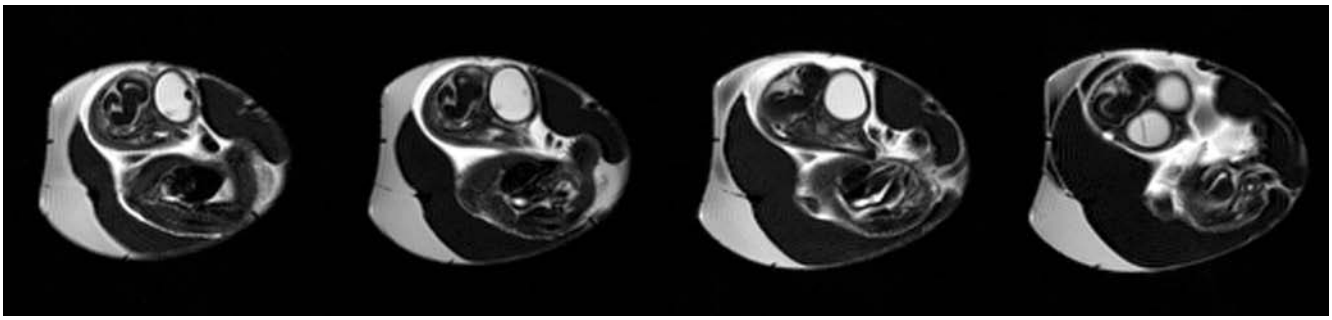


Figure 2: Four successive slices from a 12 slice single-shot RARE dataset of a live, un-anaesthetized E10 quail embryo *in ovo* acquired in 12.5 seconds.

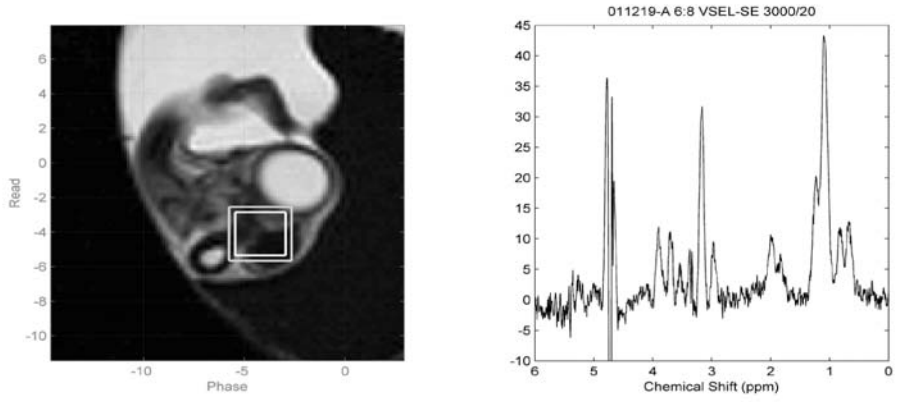


Figure 3: Single-voxel ^{1}H PRESS spectrum from the brain of an anesthetized E8 quail embryo demonstrating tentative assignments to creatine, choline and myoinositol resonances at 2.99ppm, 3.17ppm and 3.36ppm, respectively. Unassigned resonances may arise from glutamine/glutamate (3.7ppm), n-acetylasparate (2.0ppm) and yolk lipids (0.5-2.5ppm).

55. Post-processing analysis of manganese-enhanced MRI (MEMRI) tract tracing data in the murine olfactory system: Evidence of multi-synaptic transport of Mn^{2+} ion to higher order structures

Robia G. Pautler, Russell E. Jacobs

Previous work has shown that manganese ion (Mn^{2+}) is taken up through voltage activated Ca^{2+} channels, transported along axons, and across synapses. Moreover, Mn^{2+} ion is a paramagnetic MRI contrast agent, causing positive contrast enhancement in tissues where it has accumulated. These combined properties allow for its use as an effective MRI detectable neuronal tract tracer. We demonstrate here, with post-processing of the MEMRI data, Mn^{2+} tract tracing in the olfactory system can be detected multiple synapses past the primary olfactory cortex. Mice treated with $MnCl_2$ exhibited positive contrast enhancement along the olfactory pathway to the level of the primary olfactory cortex as visualized by eye. However, using the visualization software, AMIRA, we were able to perform detailed 3D analysis of the images. The histogram of the signal intensities in the MEMRI data is shown in **(Figure 1)**. Signal from un-enhanced brain tissue fell into the range of 80 – 120 on a scale of 0 – 255. Mn^{2+}-enhanced brain structures, however, fell into the top 25% of the signal intensities. From the histogram, it was possible to assign a specific color to the brain tissues that fell into this upper 25% range **(Figure 1)**. Visualization of the histogram analysis revealed tract tracing that led from the primary olfactory cortex to the entorhinal cortex as well as to the hippocampus **(Figure 2)**. Additionally, we were able to visualize connections beyond the superior colliculus in visual system tracings in the mouse **(Figure 3)**. We have demonstrated that post-processing of the Mn^{2+} tract-tracing data reveals tract tracings that exist beyond the level that is detectable by eye in unprocessed MEMRI images. Because at high concentrations, Mn^{2+} is known to be a neurotoxin, incorporation of this form of post-processing should allow for the absolute minimal amounts of Mn^{2+} to be utilized. Applications of this proposed post-processing methodology to other neuronal pathways should be very straightforward.

Figure 1

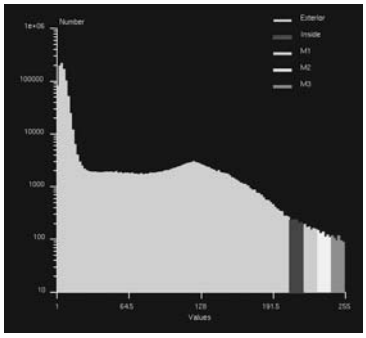


Figure 2

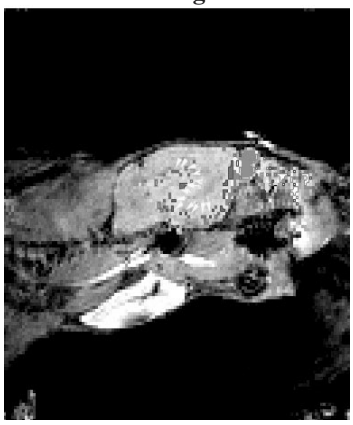


Figure 3

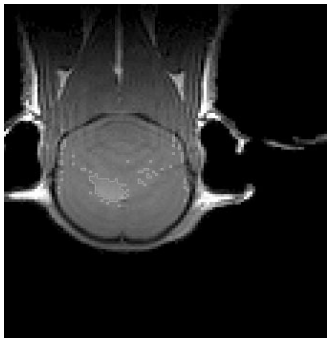


Figure 1: Histogram analysis of the mouse brain. The un-enhanced brain structures fell into the range of 80 – 120 on a scale of 0 – 255. The Mn^{2+}-enhanced brain structures fell into the top 25% of the histogram.

Figure 2: *In vivo*, sagittal section depicting the Mn^{2+} tract tracing beyond the murine primary olfactory cortex to the entorhinal cortex and hippocampus. Arrowheads, in white, point to the tract leading from the primary olfactory cortex to the entorhinal cortex and to the hippocampus.

Figure 3: *In vivo*, horizontal section depicting the Mn^{2+} tracing from the superior colliculus to the visual cortex in a mouse brain.

56. *In vivo*, dynamic Mn^{2+}-enhanced MRI (MEMRI) tract tracing along white matter tracts

Robia G. Pautler, Russell E. Jacobs

Olfactory transduction begins at the level of the olfactory receptor neurons located within the olfactory epithelium. The olfactory neurons project through the ethmoid bone of the cribiform plate where they converge to form 1 of 15 – 20 fasicles, which then project to the olfactory bulb. We demonstrate here the dynamic accumulation of Mn^{2+} along the olfactory fasicles using MEMRI. At the start of imaging (54 minutes. post Mn^{2+} lavage), the olfactory bulbs were un-enhanced. A series of images were collected for three hours post Mn^{2+} administration. During this time, enhancement within the olfactory bulbs was observed. However, at 80, 84 and 88 minutes after the start of imaging, it was possible to view Mn^{2+} traveling up the olfactory fasicles (**Figure 1 a,b**; **Figure 2**). In the time course of eight minutes, there was a 20% increase in signal intensity within the olfactory fasicle (**Figure 2**). We have demonstrated that it is possible to observe the dynamics of Mn^{2+} tract tracing *in vivo* with a

temporal resolution on the scale of minutes. The fact that the Mn^{2+} traversed so quickly in the olfactory fasicle indicates that the Mn^{2+} ion is being transported via fast axonal transport. With higher resolution scans, potential applications of MEMRI dynamic imaging include *in vivo* monitoring of the dynamics of dendritic structures during CNS development in a learning/memory paradigm. Current methodologies allow for *in vitro* work to view dynamic plasticity in the CNS in whole mount slices.

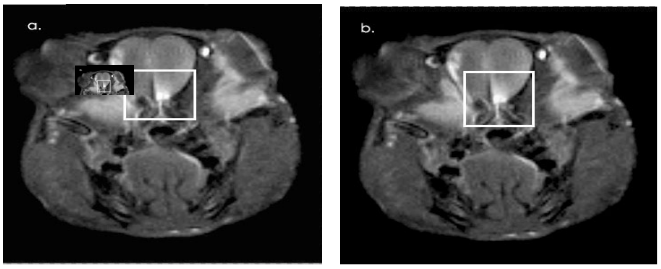
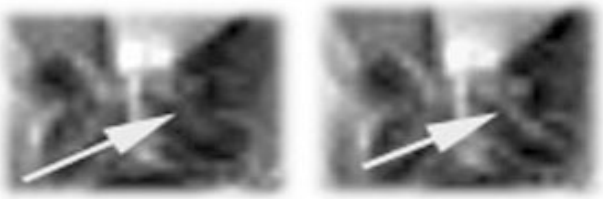


Figure 1: *In vivo*, axial images acquired at (a) 80 minutes and (b) 88 minutes after the start of imaging. The boxed areas depict the areas where the turbinates and olfactory fasicles are located.



a. **b.**

Figure 2: Blow up of boxed areas from Figure 1a/b. *In vivo*, axial images acquired at (a) 80 minutes and (b) 88 minutes after the start of imaging. Note the contrast enhancement of the olfactory fasicle in (b). In this 8 minutes time course, there was a 20% increase in signal intensity of the olfactory fasicle.

57. 3D time-apse analysis of Spemann Organizer dynamics using µMRI

Cyrus Papan, S. Sendhil Velan, Scott E. Fraser, Russell E. Jacobs

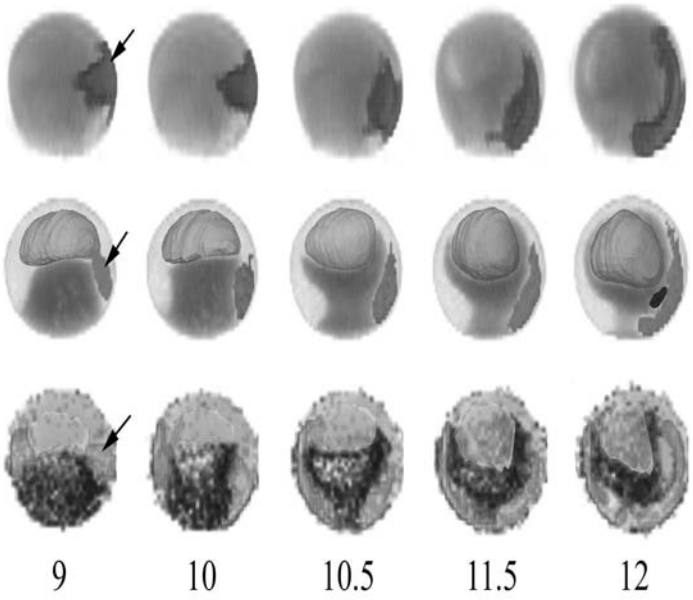
A primary function of Spemann organizer in *Xenopus laevis* is neural induction. In recent years, much insight has been gained into the molecular correlation of this process. Many aspects of this induction event have a clear prediction about the spatial relations and morphogenetic behavior of the tissues involved. However, models about tissue interactions are controversial. Because of the total opacity of the *Xenopus* embryo, it is not possible to track morphogenetic movements by optical methods. Thus, none of these relationships have been observed *in vivo*.

We seek to define the dynamics of Spemann organizer and its spatial relationship with its surrounding tissue during late blastula and gastrula stages. To overcome the problem of opacity, we have utilized NMR imaging to conduct a 3D time-lapse analysis of the morphogenetic movements of the live developing *Xenopus* embryo. Intrinsic image contrast and resolution allow us to identify the blastocoel, archenteron, and the blastopore, and to distinguish between animal and vegetal tissue. Single cells can be labeled by microinjection of contrast agent. Dynamics of clone movement can then be followed in 3D-time lapse series within the context of the whole embryo.

By labeling the C1-blastomere alone, which gives rise to most of the organizer tissue, or together with the C4 blastomere, our imaging experiments allow us to identify the relationship between the organizer and ectodermal and endodermal tissue during blastula and gastrula stages. In summary our results show that the axial mesoderm, which is considered to be in a planar configuration to the presumptive neuroectoderm, is actually vertically underlying it throughout blastula and gastrula stages. This finding resolves the long lasting controversy on the planar vs. vertical mechanism of neural induction in *Xenopus laevis.*

Dynamics of the C1-clone (arrow-below) during gastrulation in *X. laevis*. Numbers denote the embryonic stages. At stage 9, the clones extend deep into the embryo. The inner part is presumptive mesoderm which during gastrulation extends along the dorsal aspect of the embryo. The outer part is presumptive ectoderm which after gastrulation is located externally at the dorsal side of the embryo and will thus form neural tissue.

Thus, the deep presumptive mesodermal and the superficial presumptive neuroectodermal parts of the clones are in vertical contact well before gastrulation begins.



58. MR imaging with intermolecular double quantum coherences

S. Sendhil Velan, P.T. Narasimhan, Russell E. Jacobs

MR microscopy is a valuable tool for neuroimaging of small animals (1). We have demonstrated the potential advantages offered by intermolecular double quantum coherences (i-DQC's) for microscopic imaging of the mouse brain. In particular, we investigated i-DQC imaging employing two approaches. In the first approach i-DQC's are filtered and the reconverted single quantum coherences (SQC's) are phase and frequency encoded. In the second approach the i-DQC's are phase encoded during their evolution period and the reconverted SQC's are frequency encoded. These two approaches are termed i-DQF and i-DQP, respectively. Phase encoding during the double-quantum evolution period, as in i-DQP is expected to yield twice the resolution obtainable from i-DQF method and this was achieved. We have developed appropriate 2D and 3D pulse schemes to obtain images based on i-DQF and i-DQP approaches (2).

The pulse sequences were implemented on a Bruker DRX Avance spectrometer (11.7T) running ParaVision software for image acquisition. All images were obtained by employing actively shielded gradients (Micro 2.5 and Mini 0.5). Following successful test experiments with water phantoms we were able to image fixed and live mouse brains. Typical images of fixed mouse brains are shown in figure 1. In order to maintain uniform in-plane resolution the phase encoding gradient strengths used in the i-DQF and SE images are twice that used in i-DQP Imaging.

Contrast-to-noise-ratio (CNR) measurements made on these images show that the i-DQP and i-DQF CNR values are comparable but different from the spin echo (SE) value. The i-DQC signals are however weak in comparison to spin echo signals and are generally about one-fifth of the SE signals. The i-DQP method developed here promises to be of value in imaging systems with limited gradient strength.

References

(1) Ahrens, E.T., Narasimhan, P.T., Nakada, T. and Jacobs, R.E. (2001) *Prog. NMR Spectroscopy* **40**:275-306.

(2) Velan, S.S., Narasimhan, P.T. and Jacobs, R.E. (2001) *J. Magn. Reson.* **152**:189-194.

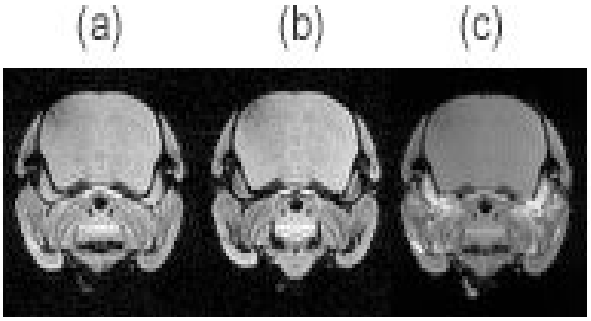


Figure 1. Three coronal slices of fixed mouse brain extracted from 3D data sets are shown. FOV = 2 x 2 x 2 cm, Matrix size = 128 x 64 x 64 (along Z, X and Y respectively). (a) i-DQP image; (b) i-DQF image; (c) SE image.

59. Design and synthesis of biochemically activated MRI contrast agents

Matthew Allen

Magnetic Resonance Imaging (MRI) is a powerful tool for noninvasive imaging of tissue and cells in biology and medicine. Contrast agents (in particular Gd(III) based-agents) enhance the observed MRI signal by altering the relaxation times of nearby water protons. Recently, "smart" contrast agents have been developed which selectively image specific biological phenomena (1-4). The systemic delivery of these agents to an organ or cell in an *in vivo* setting represents an enormous challenge. My project centers around furthering the understanding of how to chemically design MRI contrast agents that will improve the knowledge of transport *in vivo*. As a result of this the advancement of MRI contrast agents as diagnostic tools for clinical use may be affected. My specific goals include:

1. The design and synthesis of MRI contrast agents that investigate transport of molecules *in vivo*. A small organic molecule known to cross the blood brain barrier (BBB) and label the β-amyloid plaques associated with Alzheimer's disease has been covalently conjugated to a MRI contrast agent with the idea that the contrast agent will take on the delivery properties of the molecule (5). *In vitro* models of the BBB will be used to test the contrast agent. If successful, the compound will be tested *in vivo*, using mice.

2. The design and synthesis of a MRI contrast agent that will permeate cell membranes. A contrast agent with conjugated to polyarginine will be synthesized such that it will be able to cross cell membranes. This agent has potential to better the ability for the direct observation of developmental events in living embryos. Unlike previous methods, where labeled cells are identified at the termination of the experiment, this technique allows the entire kinship relationships of a clone to be determined.

Successful completion of these aims will lead to an understanding of *in vivo* activation of MRI contrast agents *in vivo*. It may also have an impact on the ability to diagnose early Alzheimer's disease.

Publications

(1) Jacobs, R.E., Ahrens, E.T., Meade, T.J. and Fraser, S.E. (1999) *TIBC* **9**:73-76.
(2) Louie, A.Y., Huber, M.M., Ahrens, E.T., Rothbacher, U., Moats, R., Jacobs, R.E., Fraser, S.E. and Meade, T.J. (2000) *Nat. Biotechnol.* **18**:321-325.
(3) Li, W.-h., Fraser, S.E. and Meade, T.J. (1999) *J. Am. Chem. Soc.* **121**:1413-1414.
(4) Moats, R.A., Fraser, S.E. and Meade, T.J. (1997) *Angew. Chem. Int. Ed. Engl.* **36**:726-728.
(5) Skovronsky, D.M., Zhang, B., Kung, M.-P., Kung, H.F., Trojanowski, J.Q., Lee, V.M.Y. (2000) *Proc. Natl. Acad. Sci. U.S.A.* **97**:7609-7614.

60. Comparison of T_2 and visual evoked potential in Shiverer and wild-type mice

Melanie Martin, Timothy D. Hiltner, Carol Readhead, Scott E. Fraser, Russell E. Jacobs

The CNS myelin sheath (essential for the rapid conduction of nerve impulses) can be disrupted for a number of reasons including: myelin genes mutations, viral infection, and autoimmune diseases. Multiple Sclerosis involves recognition of myelin proteins by T-cells followed by a cascade of destructive actions by cells of the immune system and is characterized by demyelination of axonal tracts and inflammation of the central nervous system. Common early signatures of MS include episodes of double vision and degradation of vision due to optic neuritis**.**

Visual evoked potentials (VEPs) are widely used to assess patients suspected of having MS. By measuring the latency of the signals recorded extracranially after a defined visual stimulus, VEPs offer a sensitive assay for detecting even sub-clinical (silent) lesions in the visual pathway. Although increased latency and broadened waveforms are taken to indicate lesions, little is known about the exact relationship among the VEPs and the sizes, locations or histories of lesions. Here we assess the role myelin dysfunction has in changing the VEPs.

T_2-weighted magnetic resonance imaging (MRI) can be used as a non-invasive diagnostic tool to determine the presence and location of myelin dysfunction in the CNS. We use longitudinal VEP latency measurements with T_2-weighted MRI to assess myelin in the CNS *in vivo.*

The dysmyelinating *shiverer* mutant mouse (*shi/shi*) has a mutation in the myelin basic protein gene (MBP) and produces little or no CNS myelin as compared to the wild type. As shown in figure 1, VEPs were measured in the wild type and *shiverer* mutant mice to determine the effect myelin content has on VEP measurements. As shown in figure 2, T_2-weighted MR images were taken of the brains of these mice to assess the changes seen on MRI due to dysmyelination. The latency of the VEP was larger, as expected, for the *shiverer* mutant because without myelinated axonal tracts nerve impulses are slowed.

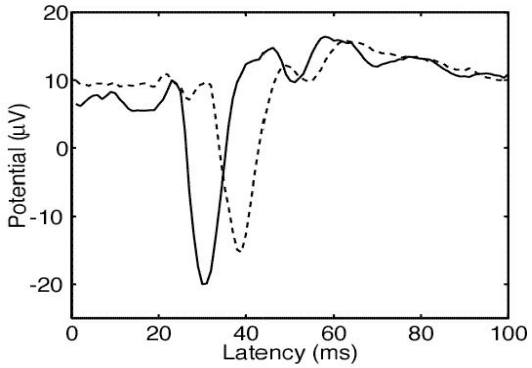


Figure 1. Visual Evoked Potentials (VEPs) from wild-type (solid) and *shiverer* (dashed) mice. This plot shows a typical VEP scan as magnitude of the VEP versus the latency, or time after the strobe light flash, for a strobe light flashing. The latency was calculated for each of 86 scans for wild type and 88 scans for *shiverer* by fitting the main peak to a Gaussian. The mean ± standard deviation of the latencies was calculated to be 30 ± 2 ms and 39 ± 1 ms for the wild-type and *shiverer* mouse, respectively. The longer latency for the Shiverer mouse is expected as nerve impulses along dysmyelinated axons are slow compared with myelinated axons.

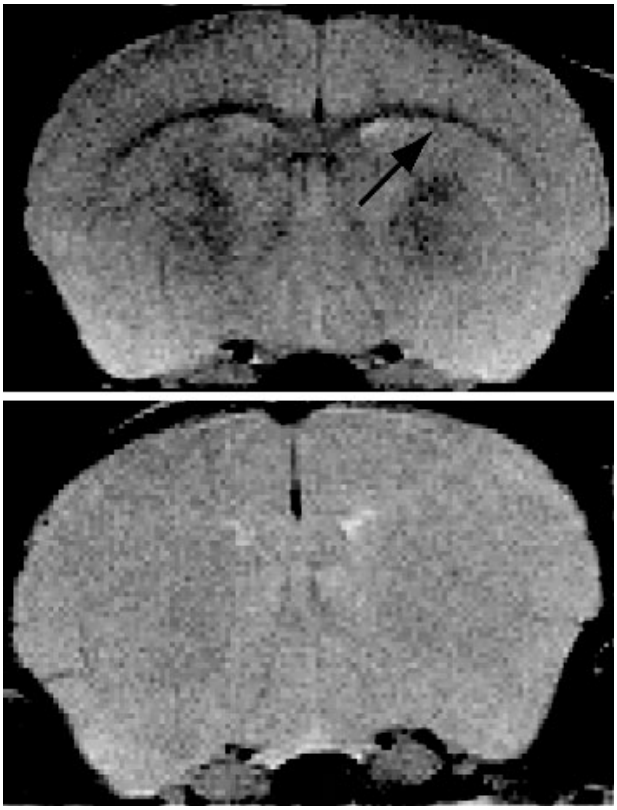


Figure 2. T_2-weighted images of wild-type (top) and *shiverer* (bottom) mouse brain. White matter appears darker in a T_2-weighted image at 11.7 T as shown by the arrow in the upper image. Notice the contrast in the image of the wild-type mouse showing the dark white matter and no contrast, indicating no myelination in the *shiverer* mouse.

61. Modeling a *Hox* gene network: Stochastic simulation with experimental perturbation

Jason C. Kastner, Jerry E. Solomon, Scott E. Fraser

The amount of molecular information that has been gathered about *Hox cis*-regulatory mechanisms allows us to take the next important step: integrating the results and constructing a higher-level model for the interaction and regulation of the *Hox* genes. Our work this past year has been focused on the investigation into a *cis*-regulatory network for the early *Hox* genes. Instead of using conventional differential equation approaches for analyzing the system, we have adopted the use of a stochastic simulation algorithm (SSA) to model the network. The model allows us to track in detail the behavior of each component of a biochemical pathway and to produce computerized movies of the time evolution of the system that is a result of the dynamic interplay of these various components. The model contains 30 different molecular species, 60 possible chemical reactions, and a typical run of the simulation consists of over 30 million chemical events. The model is able to reproduce key features of the wild-type pattern of gene expression, and *in silico* experiments yield results similar to their corresponding *in vivo* experiments. This analysis shows the utility of using stochastic methods to model biochemical networks.

Retinoic acid (RA) is an important regulator of the *Hox* genes in the aforementioned network, and despite a great deal of investigation into the connection of RA to the *Hox* genes, there are still a variety of open questions. An *in ovo* experiment is underway to test an important element of the model, namely the response of *HoxA1* to Retinoic Acid. Retinoic acid soaked beads are implanted in the mid and hindbrain of young chick embryos, and an examination of the timing and intensity of the *HoxA1* gene expression is yielding new insight into the connection. The information gathered is being used to fine tune aspects of the model, thus yielding a tight connection between the experiments and the model.

Publications

Kastner, J., Solomon, J. and Fraser, S. (2002) *Dev. Biol.* **246**:122-131.

62. TRELIS: A user-friendly Gillespie simulator for modeling chemical reaction systems

Luigi Warren, S.E. Fraser, J. Solomon

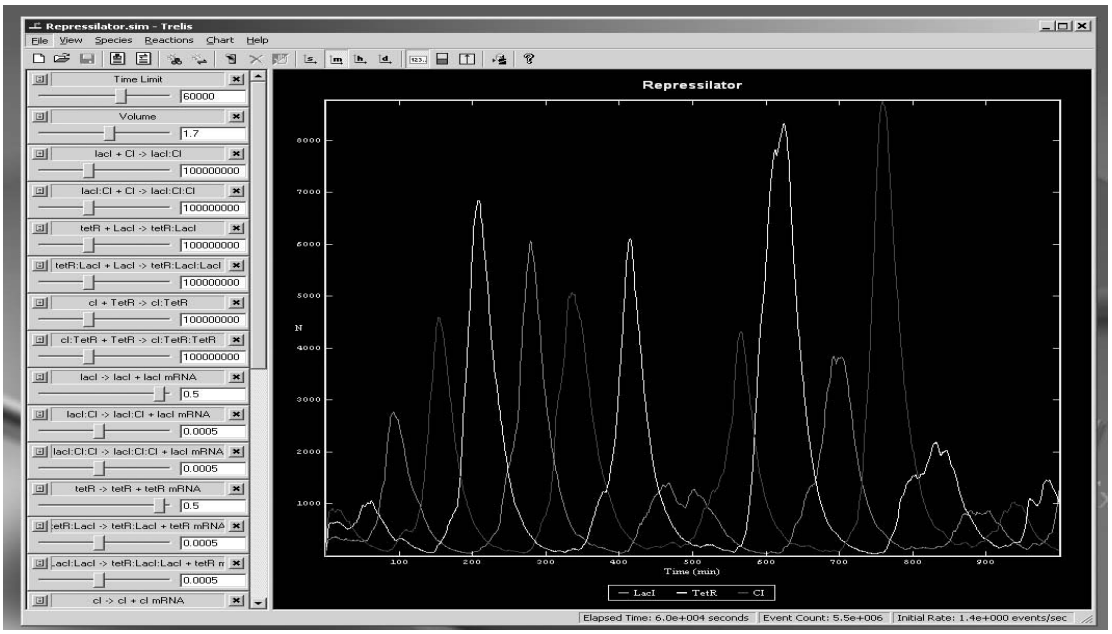
Systems biologists are interested in modeling processes occurring within the cell or at the level of fields of cells, such as signal transduction cascades, the establishment of morphogenetic gradients, and the time evolution of transcriptional regulatory networks. Most workers have adopted a deterministic modeling paradigm inherited from computational chemistry, where the behavior of the reaction system is described by a set of ordinary differential equations. This approach is highly efficient for systems involving large numbers of reacting molecules since the behavior of huge ensembles of molecules is represented by a few macroscopic variables, i.e., reactant concentrations and reaction rates.

Traditional ODE modeling does not capture the effects of stochastic noise which come to the fore in reaction systems with small numbers of molecules and low reaction rates, such as networks of cross-regulating transcription factors. It is reasonable to suppose that stochastic noise plays an important role in determining the structure of such biological circuits, both as a design constraint favoring quasi-Boolean switching behavior, and as a source for the symmetry-breaking events crucial to differentiation and development. At the same time, a formalism which focuses on reaction events rather than aggregate behavior is arguably more intuitive for modeling the granular, low-copy number chemical circuits found at the sub-cellular level, while the low event rates in such systems means they can be modeled discretely without taxing our computational resources.

A discrete, stochastic formulation of chemical kinetics has been derived by Gillespie, and serves as the basis for a class of Monte Carlo-style numerical simulations that have drawn the attention of a number of workers in the field of systems biology. In Gillespie's algorithm, the state of the reaction system is modeled as a vector representing an inventory of each molecular species in the system. The time evolution of the system is modeled by an iterative process involving two random-number draws per turn. The first draw is used to pick the time interval to the next reaction event, based on the total reaction probability given the current reactant inventory and size of the reaction compartment. The second draw is used to choose a specific reaction, based on the relative probabilities of each reaction type. The state vector is updated, decrementing the counts for reactants and incrementing the count for the products of the chosen reaction, and any affected reaction probabilities are recomputed for the next go around. In general, every simulation run produces a different stochastic trajectory. The effect of noise on the stability of the system will be apparent from a comparison of multiple simulation runs.

We have developed a new computer program based on the Gillespie algorithm to make it easy for non-programmers to build models of reaction systems, tweak their parameters, and view their time evolution graphically. TRELIS (for Transcriptional REgulation Logic In Silico) is an MFC-based Windows application. A configurable array of slider controls supports interactive, step-wise tweaking of reaction parameters. TRELIS documents save not only the contents of a model, but also the configuration of the user interface. Although the document format is unique to the TRELIS application, a new version of the program which has the ability to import and export SBML (Systems Biology Markup Language) model files will be available shortly. We plan to make the TRELIS source code available on the web in the near future.

(Photo on next page.)



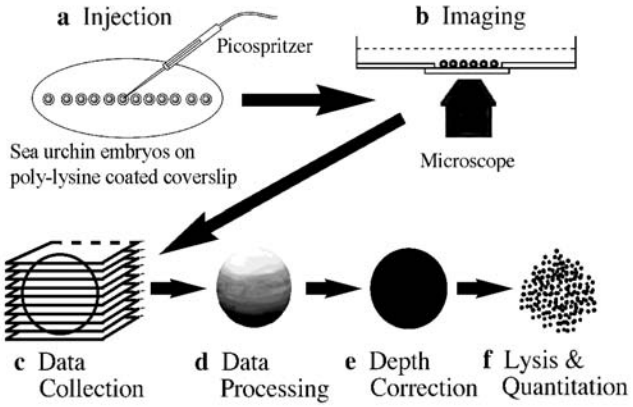
63. Quantitative imaging of *cis*-regulatory reporters in living embryos

Ivan J. Dmochowski, Jane E. Dmochowski[*]*, Paola Oliveri, Eric H. Davidson, Scott E. Fraser*

Gene transfer studies have provided a powerful vehicle for exploring the relationship between DNA sequence and gene function in a variety of living organisms. However, current analytical methods are destructive to living samples, and therefore cannot quantify gene products in both space and time. *In situ* hybridization, for example, localizes mRNA expression only in fixed specimens, making it impossible to monitor gene expression levels within the same embryo at different times. For studying dynamic gene activity in individual cells, green fluorescent protein (GFP) has distinct advantages. However, limitations in standard detection techniques and light scattering from tissue make it challenging to use GFP for quantitative analysis of gene expression in many systems (1,2). Even in sea urchin embryos, model organisms noted for their optical transparency, losses in fluorescence intensity can make internal structures of the embryo invisible. Thus, ongoing efforts to establish networks of genes, and specifically, comparisons of the activities of promoter/enhancer regions of genes transcribed during sea urchin embryogenesis (3,4), often rely on *in situ* hybridization or biochemical analyses of homogenates of several embryos.

Recently, we have developed a confocal laser scanning microscopy (CLSM) method (see Figure) for the quantitation of green fluorescent protein (GFP) as a reporter of gene activity in living three-dimensional structures, such as sea urchin and starfish embryos. This method is between two and 50 times more accurate than conventional confocal microscopy procedures, depending on the localization of GFP within an embryo. Using co-injected Texas Red dextran as an internal fluorescent standard, the observed GFP intensity is corrected for variations in laser excitation and fluorescence collection efficiency. To relate the recorded image intensity to the number of GFP molecules, the embryos were gently lysed and a fluorometric analysis of their contents was performed. CLSM data collection from a single sea urchin blastula required less than two minutes, thereby allowing gene expression in dozens of embryos to be monitored in parallel with high spatial and temporal resolution.

[*]*Division of Geological and Planetary Sciences, Caltech*



References

(1) Ferrer-Martinez, A. and Gomez-Foix, A.M. (2002) *BioTechniques* **32**:62-66.

(2) Kam, Z., Hanser, B., Gustafsson, M.G.L., Agard, D.A. and Sedat, J.W. (2001) *Proc. Natl. Acad. Sci. USA* **98**:3790-3795.

(3) Davidson, E.H., Rast, J.P., Oliveri, P., Ransick, A., Calestani, C., Yuh, C.-H., Minokawa, T., Amore, G., Hinman, V., Arena-Mena, C., Otim, O., Brown, C.T., Livi, C.B., Lee, P.Y., Revilla, R., Rust, A.G. and Pan, Z.J. (2002) *Science* **295**:1669-1678.

(4) Yuh, C.-H., Bolouri, H. and Davidson, E.H. (1998) *Science* **279**:1896-1902.

64. Guiding migrating neuronal precursors through the developing cerebellum

Reinhard W. Köester, Scott E. Fraser

The functional properties of individual brain regions are created during the structural organization of the embryonic brain. This is especially pronounced in the cerebellum that is composed of distinct functional modules such as neuronal layers, input and output nuclei. Neuronal positioning in the cerebellum is achieved during embryogenesis by long distance migration of neuronal precursors along specific migratory pathways to particular destinations. Such migratory neuronal precursors arise in a neuroepithelium running along the dorso-anterior hindbrain, the upper rhombic lip. We recently showed by *in vivo* time-lapse confocal imaging of zebrafish embryos that cerebellar neuronal precursors leaving this upper rhombic lip do not migrate exclusively in a dorsal direction as previously thought [1]. Instead, the majority of these neuronal precursors follow an antero-ventral pathway, initially toward the mid-hindbrain boundary. Here, they turn to migrate ventrally along the mid-hindbrain boundary to ventral positions underneath the cerebellum where the precerebellar nuclei are localized.

High magnification analysis of individual upper rhombic lip descendants revealed their migratory behavior, allowing conclusions about potential candidate molecules that are involved in governing these processes. By combining stable transgenesis and mutant analysis with *in vivo* time-lapse imaging, we found that proper ventral migration of upper rhombic lip-derived neuronal precursors is impaired in zebrafish mutants lacking the floorplate tissue. This indicates that guidance cues for upper rhombic lip descendants emanate from ventral midline tissue. Current molecular *in vivo* studies point at Netrin/DCC-signaling as the responsible signal transduction cascade to guide upper rhombic lip-derived neuronal precursors to ventral positions in the developing cerebellar anlage.

Publication

[1] Köester, R.W. and Fraser, S.E (2001) *Curr. Biol.* **11**:1858-1863.

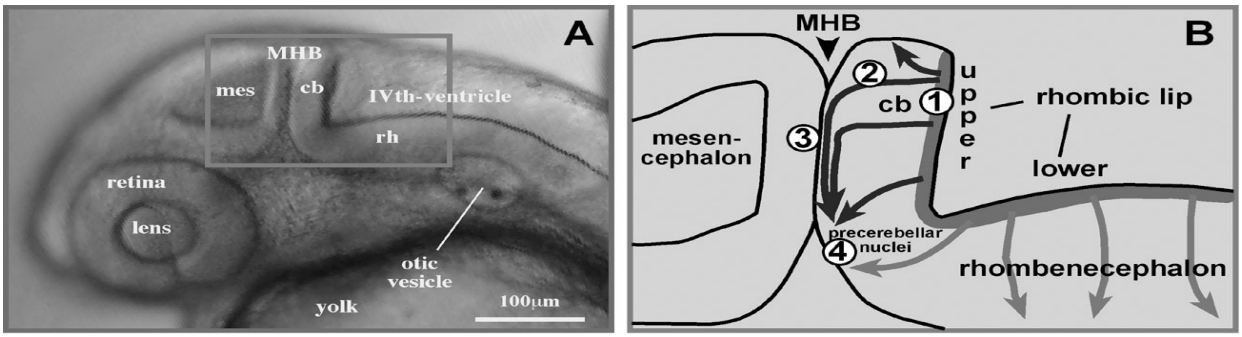


Figure 1: Schematic representation of migratory pathways originating from the rhombic lip. (A) Lateral view of zebrafish head at 24hpf (note the transparency of the tissue), the region of the anlage that will give rise to the cerebellum has been marked with a red box. (B) The majority of neuronal precursors from the upper rhombic lip migrates along an antero-ventral pathway along the mid-hindbrain boundary (MHB). Four different phases (marked 1-4) of migration can be distinguished: (1) Onset of migration involving cell polarity decisions; (2) Anterior migration toward the MHB; (3) Ventral migration along the MHB; (4) Stop of migration followed by final differentiation. Abbreviation: cb: cerebellar anlage; mes: mesencephalon; MHB: mid-hindbrain boundary; rh: rhombencephalon.

65. Fluid forces represent an essentialepigenetic factor for embryonic cardiogenesis

Jay R. Hove , Reinhard W. Köester*‡, Arian S. Forouhar* , Gabriel Acevedo-Bolton* , Scott E. Fraser‡, Morteza Gharib**

Fluid forces are known to influence cell and tissue morphology of cultured endothelial cells. To link these *in vitro* data to the intact heart, quantitative *in vivo* analysis of intracardiac flow-forces is needed. Using confocal microscopy and high-speed real time cine imaging we have measured the flow patterns, ejection fractions and erythrocyte velocities during the heart beat cycle of zebrafish embryos at different developmental stages. These studies revealed the presence of high-shear, vortical flow in the developing zebrafish heart with shear forces significantly above the cellular threshold for shear-force sensitivity even prior to cardiac valve development. These flow-forces are much greater than might be expected for a micro-scale structure (that would fit comfortably into a human hair) at such extremely low Reynolds numbers.

To test whether these shear forces are relevant for proper embryonic cardiogenesis, we occluded blood flow either into or out of the zebrafish heart by microsurgery. Zebrafish embryos are known to not being dependent on oxygen or nutrient transport through the blood during their first days of development and control experiments showed that the surgical procedure has no influence on cardiogenesis. In contrast the perturbations of blood flow resulted in hearts with an abnormal third chamber, diminished looping and impaired valve formation, indicating that the genetic mechanisms of these processes are tightly linked to intracardiac fluid forces. The similarity of these defects to those observed in some

congenital heart diseases argues for the importance of intracardiac hemodynamics as a key epigenetic factor in embryonic cardiogenesis.

Options of Bioengineering and Aeronautics, Division of Engineering and Applied Science, Caltech
‡Biological Imaging Center, Division of Biology
**Equally contributing authors*

66. *In vivo* imaging of commissural axon behavior during commissure formation in the forebrain in zebrafish

Magdalena Bak, Scott E. Fraser

We are interested in studying the early phases of forebrain circuitry development in zebrafish embryos. We would like to understand how the primary neuronal scaffold is formed, first by studying axon behaviors during commissure formation and second by gaining insight into molecular signals that guide this process.

To permit reproducible *in vivo* imaging, we employ a transgenic line (gata2::GFP) with GFP expression in the earliest differentiating cells. Using confocal microscopy, we examined GFP expression in zebrafish forebrain with respect to acetylated alpha tubulin which has been previously used to reveal the early neuronal scaffold in zebrafish. Colocalization of GFP and acetylated alpha tubulin antibody in the same cells showed that GFP positive cells in the forebrain belong to two bilateral ventral clusters of neurons.

Studies of track formation in invertebrate nervous systems have shown that a small number of early neurons, also known as pioneers, lay down an axonal scaffold that later axons and their growth cones follow. Using time-lapse fluorescence microscopy, we performed a detailed intravital characterization of forebrain commissure development and axon behavior in order to examine the behaviors of early axons and later axons that grow along them. Quantitative analysis of this dynamic process reveals drastically different behaviors for initial axons and later-growing axons at the midline. Growth cone analysis shows distinct differences between leader and follower axons as their width/length ratios as well as number and orientation of filopodia significantly differ. Our dynamic and quantitative analysis of commissural axon behaviors *in vivo*, combined with early axon ablations, shows that while all axons have the potential to act as pioneers, these in turn display behaviors specific to their leading role during neuronal track formation. In cases where the initial axon is removed, a later axon switches its behavior and acts as a pioneer.

The zebrafish forebrain primary neuron scaffold represents a simple *in vivo* model system for understanding how neuronal circuitry is built. Our results provide valuable insights into this process. As zebrafish offer the advantage of combining genetics with time-lapse imaging, our next goal is to combine our work on axon behavior(s) with analysis of the molecular guidance cues involved in axon guidance at the mid line in the forebrain.

67. Imaging analysis of cell movement patterns during zebrafish gastrulation

Ying Gong, Scott Fraser

Early vertebrate embryogenesis is characterized by extensive morphogenetic movements. During gastrulation, massive cell movements and rearrangements establish the architecture of the embryonic body by transforming a spherical egg into an embryo with three germ layers and a distinct antero-posterior axis. Due to the large number of cells involved, it has been a challenge to dissect the machinery driving these movements and to pinpoint problems when normal movements are disturbed. Therefore, we are interested in developing a method that enables us to analyze large-scale cell movements and extract patterns, and applying the method to characterize mutants with morphogenetic defects during gastrulation.

We are using zebrafish as our model system. The optical clarity of zebrafish embryos allows the movements of individual cells to be followed *in vivo*. Our previous work has established the conditions for vital dye labeling and *in vivo* imaging. By intracellular injection of mRNA encoding a fusion of histon2B and GFP, we are able to label the nuclei of a large number of gastrula cells. These nuclei are then followed *in vivo* using confocal laser scanning microscopy. Our imaging techniques have allowed us to minimize photo damage to labeled cells and collect high-resolution 4D (x, y, z, and time) images with a high sampling rate for the entire gastrulation period. These 4D images from wild-type embryos, embryos carrying a mutation in the *silberblick* (wnt11) gene, and embryos injected with *strabismus* anti-sense morpholino are imported into Volocity (Improvision Inc.). Volocity detects nuclei in the images by thresholding, determines the 3D centroid coordinates, and performs tracking in 3D object space. We are now developing Matlab functions to compare cell movement speed and direction in 3D between wild-type and mutant embryos.

68. Development of the avian enteric nervous system and gastrointestinal tract

Helen J. McBride, Scott E. Fraser

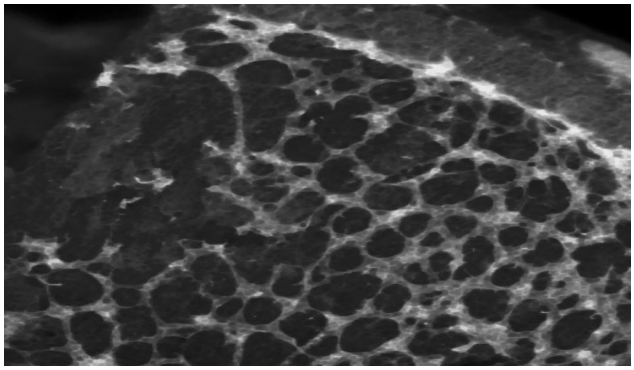
I am interested in the development of the enteric nervous system (ENS) and the surrounding caudal intestine. The model system I have chosen is the chicken, because of the easy access to embryos and the imaging techniques available for this organism. Neural crest cells form all the cells of the intrinsic ENS and do so through migration into and colonization of the entire gut. While neural crest cells are migrating, the gut tissue around them is also developing and differentiating into functional zones such as the small intestine and colon.

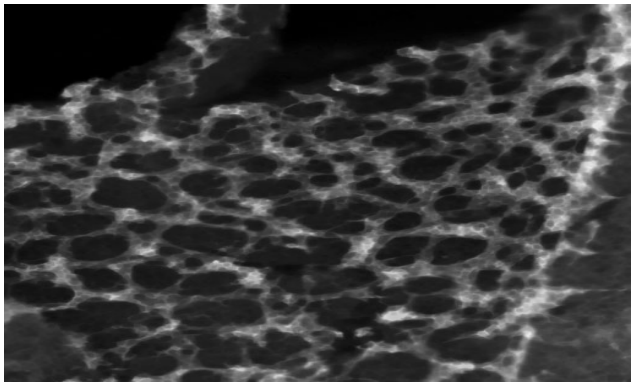
What signaling pathways are used during early gut development to make regions of the gut different from one another? I am currently investigating the contribution of the Wnt pathway to caudal gut development. Wnts and their Frizzled receptors are expressed in dynamic patterns throughout the period of gut development I am interested in and appear to be involved in growth and differentiation of the tissue. I am currently testing this hypothesis by

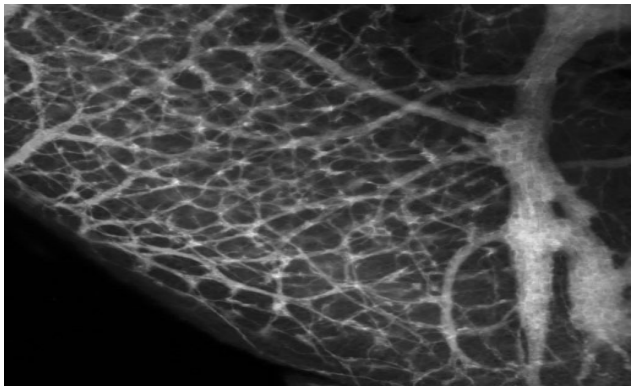
infecting early endoderm with retroviruses expressing components of the Wnt signaling pathway to perturb their function and assay for the effects on gut development.

To study the ENS, I am currently using a replication incompetent retrovirus to label neural crest cells and follow them during colonization of the gut tract. Viruses produced in our laboratory utilize GFP (Green Fluorescent Protein) localized to different parts of the cell such as the nucleus or cell membrane to make it easier to determine cell morphology as cells are migrating. By combining dynamic imaging methods and gut culture *in vitro*, I am determining what behaviors cells exhibit as they are migrating within the gut tract and beginning to differentiate into a primitive neural network.

The goal of this work is to combine the two distinct projects on ENS and gut development. I want to understand how the gut environment interacts with the early ENS cells and how these interactions influence the establishment of the primary network of the ENS.







69. The molecular control of cell movements in early vertebrate embryos

Andrew Ewald, Scott E. Fraser

The early development of vertebrate embryos is characterized by massive, coordinated cell movements. These movements shape the embryo, distribute different cell types, shape complex tissues, and bring tissues into their correct spatial relationships. The dorsal mesoderm of the frog embryo provides us with a model for the coordinated movement of connected sheets of cells. Our second model system, neural crest cells in the chicken embryo, allows us to study the migration of individual cells through a growing embryo. We have developed assays to study these models in quantitative detail and are seeking to understand the mechanisms used to coordinate these distinct types of cell movement.

The dorsal mesoderm in the frog embryo moves as a sheet of cells, due to strong connections among the cells. Cell intercalation within this sheet drives the elongation of the embryo during the process of gastrulation, whereby the round, morphologically symmetric early embryo is converted into a tadpole. We have demonstrated the existence of propagating intercellular waves of calcium within the dorsal mesoderm during gastrulation. These waves appear to be specific to the dorsal mesoderm and directly required for the cell movements of gastrulation. To build an integrated picture of how different signaling pathways interact to control gastrulation, we have developed a novel means of quantitatively imaging whole embryos with subcellular resolution. We are using this increased resolution to carefully analyze the effects of experimental perturbations on the processes of gastrulation and neurulation.

The neural crest is a transient population of cells in the vertebrate embryo that arises in the neural tube and migrates to give rise to neurons, glia, bone and other cell types. During migration individual neural crest cells make extensive temporary connections with other cells, but migrate as individuals, rather than as a connected sheet. We have used patterned substrates and optical tweezers to present them with carefully controlled molecular stimuli. We have characterized normal cellular behaviors and are characterizing the signal transduction elements necessary to generate these behaviors. These *in vitro* experiments then serve as the basis for perturbation experiments in the intact embryo.

By studying the molecular signaling required to organize two distinct types of cell movements we have learned much about the biology of these two systems. By comparing and contrasting these two systems we are learning broader lessons about the ways in which cell movements are specified across vertebrates.

70. Cranial neural crest migration in the avian embryo

Carole Lu, Paul Kulesa, Scott Fraser

The neural crest is a migratory multipotent population of cells that form derivatives in the peripheral nervous system and craniofacial structures. In avian

embryos, hindbrain neural crest cells migrate in three discrete streams adjacent to the even numbered rhombomeres r2, r4, and r6 to the branchial arches in a segmented pattern. Some believe that the segmental nature of their origin contributes to this pattern. Neural crest cells from r3 and r5 join neighbors from the even numbered rhombomeres. Work in our lab and other labs have pointed to the role of extrinsic factors, such as repulsive factors in the crest free zones adjacent to r3 and r5. In avian embryos, cranial neural crest cells migrate subectodermally and are highly amenable to *in vivo* imaging.

By using focal application of lipophilic dyes and intravital imaging, we examine the interactions between subpopulations of neural crest as they migrate together in one migratory stream. Embryological manipulations in the form of foil barriers have been used to probe the ability of neural crest cells to find their way to their final destination. Small pieces of foil barriers are placed adjacent to r4 in 9 somite-stage chick embryos to block the r4 stream of migratory neural crest cells as they emerge from the neural tube. Varying levels of blockage up to 100% will still allow the r4 stream of neural crest cells to migrate to their correct target. Cells migrate around (anteriorly or posteriorly) the barrier or over the barrier accompanied by growth of surrounding tissue. Time-lapse methods are being used to examine and quantify the individual cell behaviors of these migratory cells as they encounter the barrier and will provide a foundation for future molecular barriers and perturbations.

Understanding the importance of cell-cell interactions and the role of cadherin adhesion molecules in this highly dynamic process will be invaluable in figuring out how the pattern of cranial neural crest migration occurs.

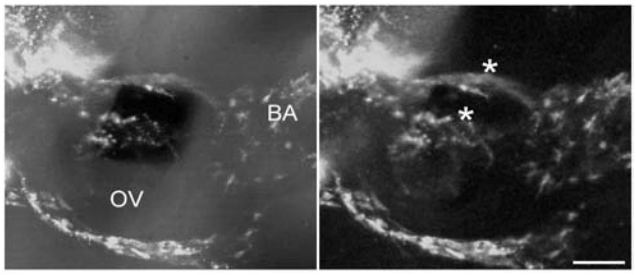


Figure 1: A typical embryo where a foil barrier blocks 93% of the region adjacent to r4, the normal pathway for the migration of the r4 stream after 18 hours. We find that the neural crest cells have been able to overcome the barrier and migrate to their proper target in two small streams (*): directly over the barrier with the help of tissue growth, and around the anterior of the barrier. OV otic vesicle, BA branchial arch. 50 μm.

71. A study of neural tube closure in mouse

P.M. Kulesa, S.E. Fraser

I am interested in the development of the neural tube, a hollow tube of nerve tissue formed by rolling up and fusing of the neural plate in a process called neurulation. The neural tube expands in the anterior to form the brain and ventricles, the narrower more posterior part forming the spinal cord. Failure of the neural plate to roll up and fuse gives rise to spina bifida and, in its most extreme form, anencephaly, both of which result in human suffering.

Although there are several mutant mouse models that display aspects of failure to close the neural tube, the analysis of specific gene function has been limited by an inability to study the effects to cell movements and tissue shaping. I have developed three objectives for this study, which if successful could offer a system to analyze normal neural tube closure and evaluate perturbations in living mouse embryos and help lead to a more complete understanding the underlying mechanisms.

First, I am studying neurulation in normal mouse embryos by developing a technique to visualize individual fluorescently-labeled cells during neural tube closure in cultured whole mouse embryos using video and confocal time-lapse microscopy. In collaboration with colleagues in our lab, we have developed a means for culturing post-implantation whole mouse embryos and I am working to extend this to focus specifically on neurulation.

My second objective is to utilize the culture and imaging techniques to analyze cell movements in a mutant mouse model (Crooked Tail) prone to exencephaly (the mouse term for anencephaly). In collaboration with Dr. Elizabeth Ross (Cornell Medical), determination of whether the primary defect is failure of the neural folds to appose or whether the folds come together and then pull apart will help guide further experiments.

Third, I plan to develop a computational model for neural tube closure that will serve as a quantitative platform to incorporate the cell movement data and for simulations of hypothetical experiments. Since at this early stage there are many possible mechanisms for neural tube closure, a computational model can help to identify crucial components of the folding and fusing process.

72. An interdisciplinary study of the genetic program underlying somitogenesis

P.M. Kulesa, S.E Fraser

Somite segmentation is one of the major foci for work on the anteroposterior patterning of vertebrate embryos, however, the lack of a system to study the temporal dynamics of cells and tissue and relationship to gene expression changes has resulted in several conflicting models. Previously, we had reported that we developed a whole chick embryo culture technique and combined intravital confocal microscopy to follow individual fluorescently-labeled cell movements during somite segmentation and a means to accurately register gene expression with the segmentation process.

Our current findings demonstrate that the sculpting of somites in chick is a more involved process than previously thought, violating current models of somite segmentation. Somitogenesis occurs in a precise spatiotemporal order of six steps: tissue separation, cell movements and integration of cells at both the anterior and

posterior somite. Somite separation is not a simple, straightforward slicing. 3D imaging of bodipy-ceramide-labeled live embryos and fixed tissue shows that a somite pulls apart from the segmental plate. Time-lapse analyses reveal this ball and socket tissue separation is followed by a series of complex movements in which cells move across the presumptive somite boundary. The movement of cells across the presumptive somite boundary is not foreshadowed by gene expression boundaries. The expression of two key genes (assumed to correlate with the site of somite boundary formation), are not expressed at the correct site to play their proposed roles. Our results motivate a model for somite formation in which both dynamic gene expression and cell motions pattern and sculpt the presomitic mesoderm into somites.

73. Dynamic *in vivo* imaging of post-implantation mammalian embryos using whole embryo culture

E.A.V. Jones, D. Crotty, P. Kulesa, C.W. Waters, M.H. Baron, S.E. Fraser, M.E. Dickinson

Due to the internal nature of mammalian development, much of the research performed is of a static nature and depends on interpolation between stages of development. This approach cannot explore the dynamic interactions that are essential for normal development. While roller culture overcomes the problem of inaccessibility of the embryo, the constant motion of the medium and embryos makes it impossible to observe and record development. We have developed a static mammalian culture system for imaging development of the mouse embryo. Using this technique, it was possible to sustain normal development for periods of 18-24 hours. The success of the culture was evaluated based on the rate of embryo turning, heart rate, somite addition, and several gross morphological features. When this technique is combined with fluorescent markers, it is possible to follow the development of specific tissues or the movement of cells. To highlight some of the strengths of this approach, we present several time-lapse movies. This first of these follows the closure of the neural tube, where the dynamic movements of the cells in the hindbrain are seen as the folds approximate in a caudal-rostral direction. A time-lapse of embryonic turning is also presented. Between 8.5 days post coital (or dpc) mouse embryos move from a ventral flexion to a dorsal flexion. This movement was followed during an 11-hour period. The third time-lapse presents somite addition in a 9.5-dpc embryo. The rate of addition matches that seen *in vivo*. The last movie highlights the strength of this technique, by combining bright field and fluorescence imaging. The time-lapse follows the development of the blood islands in a 7.5-dpc embryo, using a mouse that expresses GFP, driven by the ε-globin promoter, in its red blood cells. This work has been submitted for publication, and these movies will be available from the Genesis (Wiley Interscience) website once published.

74. Measurement of blood velocity in post-implantation mouse embryos using confocal line scanning

E.A.V. Jones, S.E. Fraser, M.E. Dickinson

The role of hemodynamics, or blood fluid dynamics, in developmental angiogenesis is controversial. Though hemodynamics are known to activate signaling pathways in mature cardiovascular systems, such as in artherosclerosis or cancer angiogenesis, the role in development has not been as intensively investigated. This is due to the limitations in measuring hemodynamics in embryos, as well as an inability to correlate hemodynamic data to changes in vessel morphology. Previous measurements of blood flow have relied on techniques such as Doppler ultrasound and Doppler echo-cardiography. Though these techniques are very useful, they often cannot resolve flows in early embryos, yet it is at these early stages of development that blood flow begins and that the most significant angiogenesis occurs (for mouse, below 10.5 days post coital, or dpc). In order to measure blood flow rates in the yolk sac of early mouse embryos, we have been using a transgenic mouse that expresses GFP in its red blood cells. This allows imaging of blood flow using fluorescence techniques. Since the flows present in the early yolk sac are, however, much too fast for conventional whole-field fluorescence or confocal microscopy, we have developed a technique to derive flow rates from individual vessels using fast line scanning. By scanning a single laser line across a vessel, rather than imaging an entire field of view, a red blood cell is imaged for longer times the slower it is moving. This results in a circular blood cells appearing as smears. Since early blood cells are nucleated and thus spherical, the dimensions of this blood cell smear can be directly correlated to the blood velocity. Using this technique, we have been able to calculate blood velocity in major and minor vessels of embryos between the age of 8.5-dpc and 10.5-dpc.

75. Using electroporation and lipid-mediated transfection of GFP-expressing plasmids to label embryonic avian cells for vital confocal and two-photon microscopy

Mary E. Dickinson, Ben A. Murray[1], Sherry M. Haynes[2], Christopher W. Waters, Kenneth J. Longmuir[2]

Fluorescent proteins have emerged as in ideal fluorescent marker for studying cell morphologies in vital systems. These proteins were first applied in whole organisms with established germ-line transformation protocols, but now it is possible to label cells with fluorescent proteins in other organisms. Our recent work has focused on using two ways to introduce GFP-expressing plasmids into avian embryos for vital confocal and two-photon imaging. First, electroporation is a powerful approach to introduce GFP into the developing neural tube, offering several advantages over dye labeling. Second, we have developed a new lipid-based transfection system for introducing plasmid DNA directly to a small group of injected cells within live, whole embryos (see

Longmuir *et al*., 2001). These complementary approaches make it possible to transfect a wide-range of cell types in the avian embryo and the bright, stable, uniform expression of GFP offer great advantages for vital fluorescence imaging. This work is reported in full in Dickinson *et al*. (2002).

[1]*Division of Biology, Caltech*
[2]*Department of Physiology and Biophysics, College of Medicine, University of California, Irvine, CA*

References

Longmuir, K.J., Haynes, S.M., Dickinson, M.E., Waring, A.J. and Murphy, J.C. (2001) *Mol. Therapy* **4**:67-74.

Dickinson, M.E, Murray, B., Haynes, S.M., Waters, C.W. and Longmuir, K.J. (2002) *Differentiation* **70**:172-180.

76. Sensitive imaging of spectrally overlapping fluorochromes using the LSM 510 META

Mary E. Dickinson, Christopher W. Waters, Gregory Bearman[1], Ralf Wolleschensky[2], Sebastian Tille[3], Scott E. Fraser

Multi-color fluorescence microscopy has become a popular way to discriminate between multiple proteins, organelles or functions in a single cell or animal and can be used to approximate the physical relationships between individual proteins within the cell, for instance, by using Fluorescence Resonance Energy Transfer (FRET). However, as researchers attempt to gain more information from single samples by using multiple dyes or fluorescent proteins (FPs), spectral overlap between emission signals can obscure the data. Signal separation using glass filters is often impractical for many dye combinations. Recently, we have developed a new, integrated laser scanning system for multispectral imaging, the Zeiss LSM 510 META (see also Dickinson *et al*., 2001). This system consists of a sensitive multispectral imager and online linear unmixing functions integrated into the system software. In order to determine the functional limits of the system, we have tested dye combinations that are heavily overlapping. The following dyes were used: GFP, DiO, Alexa 488, Fluorescein, Oregon Green, Sytox Green and ToPro-1. All of these dyes have emission peaks that fall between 500 and 550 nm. Using linear unmixing, we have been able to separate many of these fluorochromes from each other. For instance, GFP and Fluorescein, DiO and ToPro-1, and Alexa 488 and ToPro-1. Even dyes with very close emission curves, with only 5 nm of separation, such as Sytox Green and Flourescein could be separated. These studies show that it is possible to expand the number of dyes used in multicolor applications. These results are reported in full in Dickinson *et al.*, 2002.

[1]*California Institute of Technology, Jet Propulsion Laboratory, 4800 Oak Grove Drive, Pasadena, CA*
[2]*Carl Zeiss Jena GmbH, Advanced Imaging Microscopy Division, Jena, D-07745*
[3]*Carl Zeiss Microimaging, Inc., Advanced Imaging Microscopy Division, One Zeiss Drive, Thornwood, NY 10594*

Publications

Ahrens, E.T., Narasimhan, P.T., Nakada, T. and Jacobs, R.E. (2002) Small animal neuroimaging using magnetic resonance microscopy. *Prog. Nucl. Mag. Reson. Sp*. **40**:275-306.

Dhenain, M., Ruffins, S., Jacobs, R.E. (2001) Three-dimensional digital mouse atlas using high resolution MRI. *Dev. Biol.* **232**:458-470.

Dickinson, M.E, Murray, B., Haynes, S.M., Waters, C.W. and Longmuir, K.J. (2002) Using electroporation and lipid-mediated transfection of GFP-expressing plasmids to label embryonic avian cells for vital confocal and two-photon microscopy. *Differentiation* **70**:172-180.

Dickinson, M.E. and Fraser, S.E. (2001) Multi-photon laser scanning microscopy and dynamic imaging in embryos. In: *Methods in Cellular Imaging*, A. Periasamy, ed., Oxford University Press, Oxford, UK.

Dickinson, M.E., Bearman, G., Tille, S., Lansford, R. and Fraser, S.E. (2001) Multi-spectral imaging and linear unmixing adds a whole new dimension to laser scanning fluorescence microscopy. *Biotechniques* **31**:1272-1279.

Dickinson, M.E., Longmuir, K.J. and Fraser, S.E. (2001) Analysis of somitogenesis using multiphoton laser scanning microscopy (MPLSM). In: *Multiphoton Microscopy in the Biomedical Sciences*, Ammasi Periasamy, Peter T. So, editors, Proceedings of the International Society for Optical Engineering (SPIE*)*. *Prog. in Biomed. Optics Imag.* **4262**:18-26.

Dickinson, M.E., Waters, C.W., Wolleschensky, R., Bearman, G., Tille, S. and Fraser, S.E (2002) Sensitive imaging of spectrally overlapping fluorochromes using the LSM 510 META. In: *Multiphoton Microscopy in the Biomedical Sciences*, Ammasi Periasamy, Peter T. So, editors, Proceedings of the International Society for Optical Engineering (SPIE). *Prog. in Biomed. Optics Imag.* In press.

Dmochowski, I.J., Dmochowski, J.E., Oliveri, P., Davidson, E.H., Fraser, S.E. (2002) Quantitative confocal laser scanning microscopy for *in vivo* genomics studies. *PNAS* **99**:12895-12900.

Ewald, A., McBride, H.J., Reddington, M., Fraser, S.E., Kerschmann, R. (2002) Surface imaging microscopy. An automated method for visualizing whole embryo samples in three dimensions at high resolution. *Dev. Dynamics*. In press.

Jacobs, R.E. and Cherry, S. (2001) Complementary emerging techniques: high-resolution PET and MRI. *Curr. Op. Neurobiol.* **11**:621-629.

Jones, E.A.V., Crotty, D., Kulesa, P.M., Waters, C.W., Baron, M.H., Fraser, S.E., Dickinson, M.E. (2002) Dynamic *in vivo* imaging of post implantation mammalian embryos using whole embryo culture. *Genesis*. In press.

Kastner, J.C., Solomon, J.E. and Fraser, S.E. (2002) Modeling a *hox* gene network in silico using a stochastic simulation algorithm. *Dev. Biol.* **246**:122-131.

Keegan, B.R., Feldman, F.L., Lee, D.H., Koos, D.S., Ho, R.K., Stainier, D.Y., Yelon, D. (2002) Spt6 and Sp5 promote transcription in the zebrafish. *Development* **129**:1623-1632.

Köster, R.W., Fraser, S.E. (2001) Direct imaging of *in vivo* neuronal migration in the developing cerebellum. *Curr. Biol.* **11**:1858-1863.

Köster, R.W., Fraser, S.E. (2001) Tracing transgene expression in living zebrafish embryos. *Dev. Biol.* **233**:329-346.

Kulesa, P.M. and Fraser, S.E. (2002) Time lapse analysis reveals cell dynamics during somite boundary formation in whole chick embryo explants. *Science*. In press.

Lansford, R., Bearman, G., Fraser, S.E. (2001) Resolution of multiple GFP color variants and dyes using two-photon microscopy and imaging spectroscopy. *J. Biomed. Optics* **6**:311-318.

Lichtman, J.W., Fraser, S.E. (2001) The neuronal naturalist: watching neurons in their native habitat. *Nature Neurosci.* **4**:1215-1220.

Longmuir, K.J., Haynes, S.M., Dickinson, M.E., Waring, A.J. and Murphy, J.C. (2001) Optimization of a peptide/non-cationic lipid gene delivery system for effective microinjection into chicken embryos *in vivo. Mol. Therapy* **4**:67-74.

Mathis, L., Kulesa, P.M. and Fraser, S.E. (2001) FGF receptor signaling is required for the maintenance of neural progenitors during Hensen's node progression. *Nat. Cell Biol*. **3**:559-566.

Moats, R., Ma, L.Q., Wajed, R., Sugiura, Y., Lazaryev, A., Tyszka, M., Jacobs, R., Fraser, S., Nelson, M.D. and DeClerck, Y.A. (2001) Magnetic resonance imaging for the evaluation of a novel metastatic orthotopic model of human neuroblastoma in immunodeficient mice. *Clin. Exp. Metastasis* **18**:455-461.

Neusch, C., Rozengurt, N., Jacobs, R.E., Lester, H.A. and Kofuji, P. (2001) Kir4.1 potassium channel subunit is crucial for oligodendrocyte development and *in vivo* myelination. *J. Neurosci.* **21**:5429-5438.

Potter, S.M., Zheng, C., Koos, D.S., Feinstein, P., Fraser, S.E., and Mombaerts, P. (2001) Structure and emergence of specific olfactory glomeruli in the mouse. *J. Neurosci*. **21**:9713-9723.

Stern, C.D. and Fraser, S.E. (2001) Tracing the lineage of tracing cell lineages. *Nature Cell Biol.* **3**:216-218.

Thakur, A., Lansford, R., Thakur, V., Narone, J.N., Atkinson, J.B., Buchmiller-Crair, T., Fraser, S,E. (2001) Gene transfer to the embryo: strategies for the delivery and expression of proteins at 48 to 56 hours postfertilization. *J. Pediatr. Surg.* **36**:1304-1307.

Tyszka, J.M. and Mamelak, A.N. (2001) Volumetric multi-shot echo-planar spectroscopic imaging. *Magn. Reson. Med*. 4**6**:219-227.

Uvadja, A.J., Köster, R.W. and Skene, J.H.P. (2001) GAP-43 promoter elements in transgenic zebrafish reveal a difference in signals for axon growth during CNS development and regeneration. *Development* **128**:1175-1182.

Wallingford, J.B., Ewald, A,J., Harland, R.M. and Fraser, S.E. (2001) Calcium signaling during convergent extension in *Xenopus*. *Curr. Biol.* **11**:652-661.

Wallingford, J.B., Fraser, S.E. and Harland, R.M. (2002) Convergent extension: The molecular control of polarized cell movement during embryonic development. *Dev. Cell* **2**:695-706.

Wolleschensky, R., Dickinson, M.E. and Fraser, S.E. (2001) Group velocity dispersion and fiber delivery in multiphoton laser scanning microscopy. In: *Confocal And Two-Photon Microscopy: Foundations, Applications and Advances*. Alberto Diaspro, ed., New York: Wiley-Liss Inc.

Associate Professor: Bruce A. Hay
Research Fellows: Peizhang Xu, Cain H. Yam, Soon Ji Yoo
Graduate Students: Jeffrey Copeland, Jolene Fernandez, Jun R. Huh, Stephanie Vernooy
Undergraduate Students: Vivian Chow
Collaborators: H.-A.J. Müller[1], M. Guo[2], Rollie Clem[3], Yigong Shi[4]
Research Staff: Asya Pogadina, Hong Yu
[1]*Heinrich-Heine Universitat, Dusseldorf, Germany*
[2]*Department of Neurology, UCLA*
[3]*Kansas State University*
[4]*Princeton University*

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Summary: We are interested in how cell fate choice is regulated and carried out. A large focus of our work is directed towards understanding the genetic and molecular mechanisms that regulate and bring about cell death. Specifically, we are using *Drosophila melanogaster* as a model system to identify genes that function to regulate cell death, and to identify important roles that cell death plays in normal development. Important cellular regulatory pathways are evolutionarily conserved; thus, molecules identified as important regulators of cell death in *Drosophila* are likely to have homologs in vertebrates and the pathways that link these molecules are likely to be regulated similarly. A second set of goals is to take the molecules and pathways uncovered in *Drosophila* and apply this information to the study of cell death in vertebrates, with the ultimate goal of determining the role that aberrations in this process play in human pathologies. In this context we see *Drosophila* as a powerful tool for uncovering conserved components and modes of death regulation. Toward this end we have developed several genetic screening approaches in the fly, and in the yeast *Saccharomyces cerevisiae* (described below), to identify genes important in the regulation of cell death.

We have also become interested in identifying and characterizing site-specific proteases, and in identifying regulators of their activity. We have developed an approach to monitoring the activity of site-specific proteases in the yeast *Saccharomyces cerevisiae*. We are using yeast that act as reporters for the activity of site-specific proteases as backgrounds in which to carry out screens for these proteases and their regulators.

Cell death

Apoptosis is a form of regulated cell death in which superfluous or harmful cells are removed from an organism. Apoptotic cell death is required for many aspects of normal development, tissue size homeostasis, and as a defense against potentially harmful cells, such as self-reactive cells in the immune system, virally-infected cells, cells that have damaged DNA, or cells that are being induced to proliferate inappropriately (reviewed in Ellis *et al*., 1991; Vaux *et al*., 1994; Steller, 1995; Jacobson *et al*., 1997). Because cell death is widespread during the development and normal function of organisms, de-regulation of this pathway has dire consequences. Inappropriate cell death is associated with degenerative neurological diseases such as Alzheimer's disease and Parkinson's disease; inhibition of normally occurring cell death can contribute to the development of auto immunity, persistent viral infections, and can set the stage for cancer by preventing the death of cells that would normally die, allowing them to undergo mutations that could lead to transformation (reviewed Thompson, 1995).

Although the signals and stimuli that trigger cell death are diverse, once initiated, apoptosis is thought to proceed via one or several common pathways. The identification in worms, flies and mammals of homologous proteins that function similarly to regulate cell death indicates that, as with other important signal transduction pathways, components and modes of death regulation are likely to be conserved throughout evolution (reviewed in Vernooy *et al*., 2000). *Drosophila* is an ideal system in which to do screens for genes important in death signaling because it is a complex organism with multiple life stages in which death plays important roles, it has powerful genetics, and we are able to manipulate death signaling in individual tissues using tissue-specific promoters.

References

Vernooy, S.Y., Copeland, J., Ghaboosi, N., Griffin, E.E., Yoo, S.J. and Hay, B.A. (2000) *J. Cell Biol*. **150**:F69-F75.
Ellis, R.E., Yuan, J.-Y. and Horvitz, H.R. (1991) *Annu. Rev. Cell Biol*. **7**:663-698.
Jacobson, M.D., Weil, M. and Raff, M.C. (1997) *Cell* **88**:347-354.
Steller, H. (1995) *Science* **267**:1445-1449.
Thompson, C.B. (1995) *Science* **267**:1456-1462.
Vaux, D.L., Haecker, G. and Strasser, A. (1994) *Cell* **76**:777-779.

The dominant modifier approach

Since normally occurring cell death is essential for *Drosophila* viability, genetic approaches to cell death must take account of this fact as well as the potentially important role of maternal effect gene products. We have circumvented these problems by creating a sensitized system that exploits a tissue dispensable for viability and fertility, the eye. In this system a cell death-signaling pathway is made hyperactive (giving rise to flies with small eyes) or partially nonfunctional (giving rise to flies with large, rough eyes). In this sensitized background a decrease in gene dosage of 50% in genes functioning downstream or in parallel to the point at which the signaling pathway has been manipulated might be expected to result in an eye phenotype change, if these components are now rate limiting.

To carry out this approach we use a P-element expression vector, pGMR (**G**lass **M**ultimer **R**eporter), which drives eye-specific expression of reporter genes (Hay, *et al*., 1994). To identify genes important in cell death regulation, proteins from *Drosophila* or other systems that are known to be able to activate or inhibit cell death in some context are expressed in the eye under GMR control, and their capacity to perturb the normal patterns of cell death determined. To the extent that the ability of these proteins to manipulate cell death is conserved, such expression should result in the creation of flies with visible phenotypes (increased cell death=small eye; decreased cell death=large, rough eye). These flies can then be used as screening backgrounds to identify interacting genes.

Several genes have been identified that influence cell death in the fly. The 75C region contains three genes *reaper* (*rpr*) (White *et al*., 1994), *hid* (Grether *et al*., 1995), and *grim*, (Chen *et al*., 1996) (known as the 75C death activators) that are necessary for most normally occurring cell death in *Drosophila*. Expression of these genes is largely (though with some exceptions) restricted to cells that have been induced to die, and expression of any of these genes is sufficient to induce the death of at least some normally living cells in which they are ectopically expressed, including cells in the eye (White *et al*., 1994; Grether *et al*., 1995; Hay *et al*., 1995; Chen *et al*., Pronk *et al*., 1996; White *et al*., 1996; Chen *et al*., 1996). Normally occuring cell death, as well as cell death due to expression of the 75C death activators, can be prevented by expression of the baculovirus protein p35, a broad specificity caspase inhibitor which blocks cell death in many organisms (reviewed in Teodoro and Branton, 1997), including *Drosophila* (Hay *et al*., 1994).

To isolate new cell death regulators we carried out loss-of-function modifier screens designed to identify enhancers and suppressers of an increase in cell death-induced small eye phenotype due to overexpression of the *Drosophila reaper* (*rpr*) gene (GMR-*rpr* flies). The premise of this screen is that a two-fold decrease in signaling strength (making the fly heterozygous at important death regulatory loci) will result in a change in eye phenotype. During this screen we identified the cell death inhibitors DIAP1 and DIAP2 (Hay *et al*., 1995), a focus of much of our current work.

References

Chen, P., Nordstrom, W., Gish, B. and Abrams, J.M. (1996) *Genes Dev*. **10**:1773-1782.

Grether, M.E., Abrams, J.M., Agapite, J., White, K. and Steller, H. (1995) *Genes Dev*. **9**:1694-1708.

Hay, B.A., Wolff, T. and Rubin, G.M. (1994) *Development* **120**:2121-2129.

Hay, B.A., Wassarman, D.A. and Rubin, G.M. (1995) *Cell* **83**:1253-1262.

Pronk, G.J., Ramer, K., Amiri, P. and Williams, L.T. (1996) *Science* **271**:808-810.

Teodoro, J.G. and Branton, P.E. (1997) *J. Virol*. **71**:1739-1746.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K. and Steller, H. (1994) *Science* **264**:677-683.

White, K., Tahaoglu, E. and Steller, H. (1996) *Science* **271**:805-807.

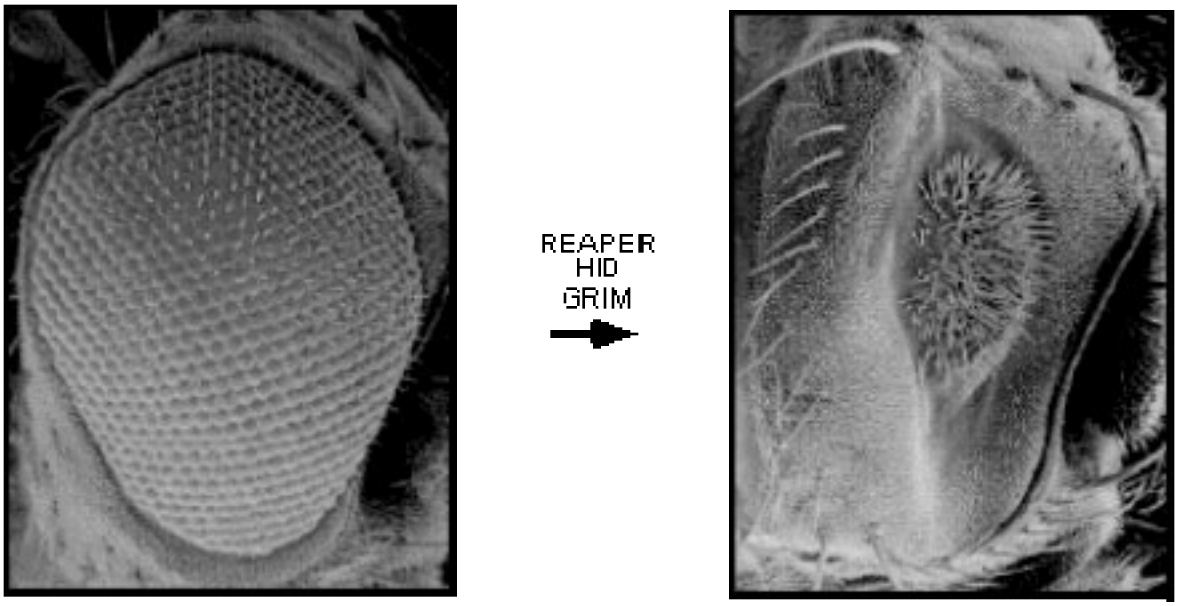

Figure 1. Overexpression of REAPER, HID or GRIM in the fly eye leads to excess cell death, which is manifested as flies with small eyes.

The Drosophila IAP family of cell death inhibitors

Overexpression of either DIAP1 or DIAP2 blocks death in response to multiple stimuli, suggesting that they act on components common to multiple death signals. Though these proteins are homologous, they are different enough from each other in sequence (30% identity), genetic interactions and expression pattern that they probably function in distinct cellular contexts and have preferred substrates of action. DIAPs have two major functional domains: an N-terminal region containing two or three BIR repeats, sufficient for death-preventing activity, and a C-terminal RING finger, which may be functioning to negatively regulate or antagonize this death-preventing activity.

As described below, *Drosophila* IAPs can interact with a number of proteins. Our evidence shows that DIAP1 is required for cell survival, and that a major function of the protein is to inhibit caspase activity. Death activators such as RPR, HID and GRIM act, at least in part, by blocking DIAP1 function, unleashing latent caspase activity. We are interested in understanding how proteins like RPR, HID and GRIM block DIAP1 function. We would also like to determine if peptides or other small molecules can mimic the function of these proteins. Because IAPs are the only known family of caspase inhibitors and, based on our observations, their function is required for cell survival, we think that modulating their ability to interact with caspases is likely to be an important mechanism by which cells can be made more or less sensitive to caspase-dependent cell death.

In other work, described below (Yoo *et al*., 2002), we have addressed control of IAP function through a second mechanism, ubiquitination. This work stems from the observation that multiple IAPs can function as E3 ubiquitin-protein ligases, and that this activity requires the C-terminal RING domain. We have found that DIAP1 autoubiquitinates, which promotes its own degradation (presumably a proapoptotic function), and it can also ubiquitinate and degrade proapoptotic proteins to which it binds, such as caspases and RPR, HID and GRIM (presumably an antiapoptotic function). Given these observations the obvious question is how is this activity regulated, and what are the contexts in which it is regulated, to play either pro- or anti-apoptotic roles?

Reference

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M.R., Clem, R.J., Muller, H.-A.J. and Hay, B.A. (2002) *Nature Cell Biol*. **4**:416-424.

Gene activation screens

Mutational inactivation is an important approach to understanding the role a gene plays in a specific process. The approach is limited, however, by the facts that many genes do not have an easily assayable loss-of-function phenotype, and that any phenotype that is observed reflects only that aspect of a gene's function that is not compensated for by other genes and pathways. An alternative approach to understanding gene function is to characterize phenotypes associated with tissue-specific misexpression of genes at elevated levels in tissues where they are normally expressed, or in tissues in which they are not normally expressed. Misexpression may create phenotypes where inactivation does not, providing a powerful approach to identifying genes. Misexpression also allows one to determine if the presence of a specific gene product is sufficient to drive a process.

To carry out misexpression screens for genes important in a particular process one needs to be able to drive the expression, individually, of large numbers of genes in a specific tissue. It is not feasible to individually misexpress known genes because this requires that one have the full-length gene in hand, that these be introduced into the genome one at a time, and that one pre-select candidate genes that are likely to be important for the process under study. Misexpression of random genes from their normal genomic location provides a much more general approach for identifying genes that can affect a process without preconceptions.

Drosophila is an ideal system in which to carry out such random misexpression screens because transposable elements (P-elements) can be mobilized throughout the genome at a high frequency, in a controlled fashion (Cooley *et al*., 1988), and because mutagenic P elements have a preference for insertion near the 5' ends of a genes (Spradling *et al*., 1995). To carry out this approach we designed a P-element vector, known as GMREP, which contains an eye-specific promoter near one P element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P-element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay *et al*., 1997). Flies carrying these insertions can then be tested in various ways to identify those that are misexpressing cell death regulators.

Below we discuss the use of the gene misexpression approach to identify novel cell death regulators. We have carried out several large screens for cell death inhibitors and have identified a modest number of interesting loci. One of these, the Bruce locus (others named it this in mammalian systems, not us), encodes a very large protein with E2 ubiquitin conjugation activity. *Drosophila* Bruce acts as a very potent inhibitor of RPR- and GRIM-dependent cell death (Vernooy *et al*., 2002). Interestingly, and consistent with a possible role of Bruce as an oncogene, mammalian Bruce is upregulated in multiple human cancers.

References

Cooley, L., Kelley, A. and Spradling, A. (1988) *Science* **239**:1121-1128.

Hay, B.A., Maile, R. and Rubin, G.M. (1997) *Proc. Natl. Acad. Sci. USA* **94**:5195-5200.

Spradling, A.C., Stern, D.M., Kiss, I., Roote, J., Laverty, T. and Rubin, G.M. (1995) *Proc. Natl. Acad. Sci. USA* **92**:10824-10830.

Vernooy, S.Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Olson, M.R. and Hay, B.A. (2002) *Curr. Biol.* **12**:1164-1168.

Yeast based screens for caspases and their regulators

Site-specific proteolysis plays a number of critical roles in regulating cellular processes. For each of these cleavage events we would like to know what the protease

is that is doing the cleavage, what the targets of the protease are, how the activity of the protease is regulated, and where and when the protease is active? A useful tool for addressing these questions would be a reporter for protease activity whose presence or absence can be visualized and quantitated. We have devised such a protease reporter that uses living cells. The basis for our approach is the creation of chimeric proteins that consist of two protein domains separated by a protease target site. One of the two domains, domain A, when released from association with domain B, is able to act as a signal transducer. Domain B functions to inhibit the signal transducing function of domain A when they are linked. Thus, in the absence of a protease that cleaves between domains A and B, no signal is transduced, while in the presence of the appropriate protease, the target site is cleaved, releasing domain A to transduce a signal which is subsequently detected.

There are a number of possible ways of constructing such a system and we have pursued several of these. One approach that works well involves creating a fusion protein that consists of a transcription factor (domain A) tethered to the intracellular domain of a type 1 plasma membrane protein (domain B). In this fusion, the C terminus of the transmembrane protein is separated from the transcription factor by a linker domain that contains protease target sites. In the absence of a protease that can cleave the target site, the transcription factor remains at the membrane where it is nonfunctional. When the target site is cleaved by an introduced or endogenous protease, the transcription factor is released from the membrane and is free to go to the nucleus and activate transcription of a reporter.

A central step in carrying out apoptosis is the activation of members of a family of cysteine-dependent, aspartate-specific proteases, known as caspases. In many cell types, caspases sufficient to carry out cell death are expressed ubiquitously, indicating that their activation and activity must be tightly controlled in normal cells. Because caspases are central to cell death regulation, they are important potential therapeutic targets. In order to take advantage of this potential, it is important to identify and characterize the organismal complement of caspases, and to understand how their activity is regulated by other cellular factors. With these goals in mind, we have developed yeast that act as reporters for caspase activity. Yeast provides an ideal background in which to screen for caspases and their regulators because: 1) They can be transformed with high efficiency, allowing for the screening of large numbers of proteins for activity in any particular assay; 2) Strains with a number of nutritional markers are available, allowing for the introduction and maintenance of a number of different plasmids at defined copy number; 3) Powerful selections can be carried out; and 4) It is likely that the intracellular environment of yeast constitutes a null, or close to null, state with respect to the activity of higher eukaryotic cell death regulators, including caspases.

Our caspase reporter yeast express a fusion protein substrate for caspase cleavage in which the type 1 transmembrane protein CD4 has linked to its cytoplasmic tail the transcription factor LexA-B42 (LB). Separating these two domains is a short linker (DG6) consisting of six different caspase cleavage sites that bracket the specificities of known caspases and the serine protease granzyme B, which cleaves caspases at sites of similar sequence (Thornberry *et al*., 1997). The emerging N terminus following cleavage at any of the caspase target sites is a glycine residue, which acts as a stabilizing residue in the N-end rule degradation pathway in yeast (Varshavsky, 1996). When this molecule, referred to as CLBDG6 is expressed in a yeast strain that carries a plasmid in which expression of the *lacZ* gene is under the control of a LexA-B42-dependent promoter (designated as the LexA/B-gal reporter strain), expression of *lacZ*, requires caspase cleavage at one or more of the introduced target sites, which releases LexA-B42 from membrane association, allowing it to activate *lacZ* transcription. Using this strain and other related strains, in conjunction with *Drosophila* and human cDNA yeast expression libraries we have generated, we are screening for caspases and caspase inhibitors (Hawkins *et al*., 1999).

The transcription-based reporter strategy to monitoring caspase activity in yeast also lends itself to screens designed to identify other site-specific proteases. One class of proteases that has generated a large amount of interest are those that cleave membrane proteins in the transmembrane domain. As described below we are pursuing screens designed to identify molecules that modulate an activity known as gamma secretase, which is critical for the cleavage of the Amyloid Precursor Protein (APP).

We also found that overexpression of an active caspase kills yeast. This fact provides the basis for very powerful set of screens for proteins that function as caspase inhibitors as molecules that block caspase-dependent cell death. As described below, we have used this screen as a tool to show that the *Drosophila* cell death inhibitor DIAP1 functions as a caspase inhibitor (Hawkins *et al*., 1999). Cells that live because they express a caspase inhibitor, as well as an active caspase, provide a background in which to identify molecules that can disrupt caspase inhibitor-caspase interactions by virtue of their ability to kill these yeast, but not naive yeast. As described below, we have used this approach, as well as *in vitro* assays using purified proteins, to show that the *Drosophila* cell death activators REAPER, HID and GRIM block DIAP1's ability inhibit caspase activity, suggesting a mechanism by which they induce cell death (Wang *et al*., 1999).

References

Thornberry, N.A. *et al*. (1997) *J. Biol. Chem*. **272**:17907-17911.

Varshavsky, A. (1996) *Proc. Natl. Acad. Sci. USA* **93**:12142-12149.

Hawkins, C.J. *et al*. (1999) *Proc. Natl. Acad. Sci. USA* **96**:2885-2890.

Wang , S.L. *et al*. (1999) *Cell* **98**:453-463.

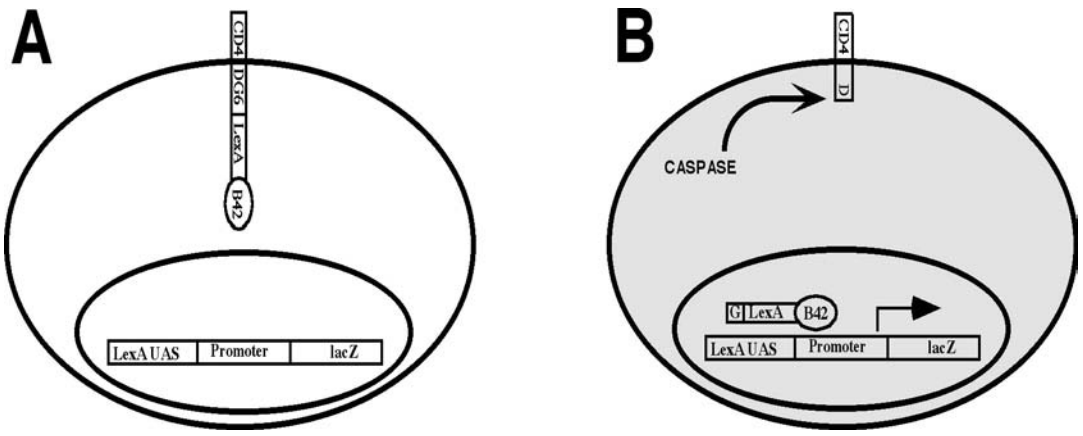

Figure 2. A yeast strain that acts as a reporter for caspase activity. (A) Six caspase cleavage sites are placed between an N-terminal fragment of CD4 and a C-terminal LexA-B42 transcription factor. (B) Caspase cleavage results in release of the LexA-B42 protein, and activation of *lacZ* transcription

77. Yeast and fly based screens for proteases that can cleave in a transmembrane environment

Ming Guo, Jolene Fernandez, Bruce A. Hay

Alzheimer's disease is genetically heterogeneous, but it is invariantly associated with the accumulation in the brains of affected individuals of senile plaques consisting largely of amyloid beta peptide (A-beta), which is derived by proteolytic processing from the amyloid precursor protein (APP). A large body of evidence suggests that A-beta deposition is a cause rather than a consequence of Alzheimer's disease. Thus, blocking A-beta deposition is an important therapeutic goal. APP is initially translated as a type 1 transmembrane protein, but it can be processed by different pathways (see **Figure 2**). In the major pathway, alpha secretase releases the APP N-terminal ectodomain into the lumenal and extracellular space. Alpha secretase cleaves in the middle of the sequence that could give rise to the A-beta peptide, thus precluding its formation. In an alternative pathway A-beta peptides are formed through the action of beta and gamma secretases. Gamma secretase activity (which may consist of distinct proteases) cleaves in the transmembrane region of APP to generate, in conjunction with beta secretase, two major A-beta species of 40 or 42-43 residues in length, differing in the length of their C termini. The longer forms of A-beta aggregate and are thought to seed the formation of amyloid plaques. The molecular nature of the gamma secretase(s) is unknown.

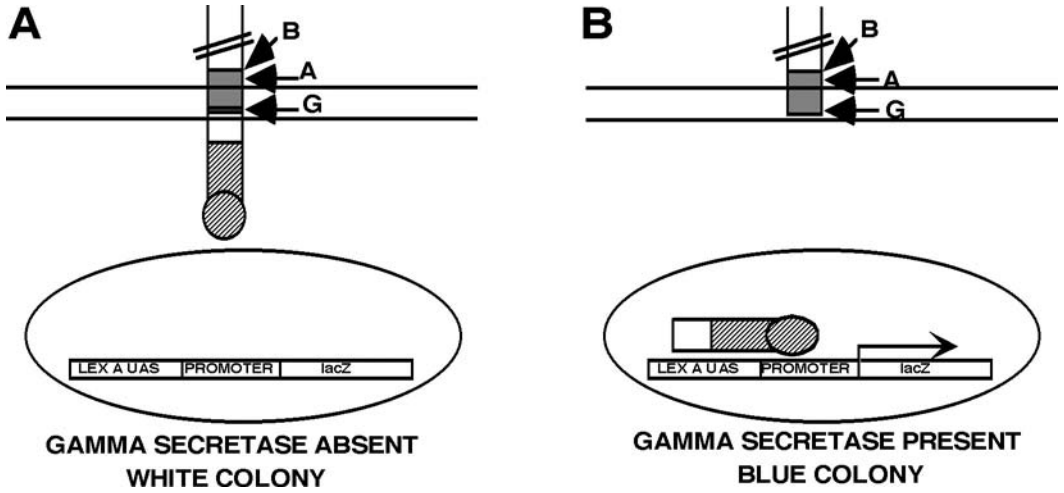

Figure 3. Generation of a yeast strain that acts as a reporter for gamma secretase activity. APP is made as a type 1 transmembrane protein. In (**A**) APP (open and shaded boxes) is shown with LexA-B42 (diagonal lined domains) fused to its C-terminus. Cleavage by alpha secretase (A) results in release of an N-terminal ectodomain. Alpha secretase cleaves within the A-beta peptide sequence (shaded box). Alternatively, cleavage by beta (B) and gamma secretase (G) results in formation of the A-beta peptide 40 and 42-43 forms and release of a C-terminal fragment consisting of the APP cytoplasmic domain. (**B**) In the presence of gamma secretase the APP cytoplasmic domain-LexA-B42 fusion is released from the membrane and translocates to the nucleus where it activates transcription of lacZ.

We have developed two screens to search for proteins that either are gamma secretase activity, or that regulate its activity. The first screen, diagrammed in **Figure 2**, is a simple variant of the caspase reporter system in yeast, in which lacZ expression is the readout. We generated a form of APP in which a cleavable signal sequence lies just N terminal to the APP beta secretase cleavage site. APP C-terminal sequences are then followed by the transcription factor LexA-B42. We are using yeast expressing this construct, as well as one of the presenilins, as a background in which to screen for proteins that show potential gamma secretase activity: cleavage-dependent reporter activation.

We have also set up a related screen in *Drosophila*, in which a similar APP fusion protein (in which the transcription factor is GAL4) is expressed in the eye in flies that carry a UAS-*rpr* construct. In this system release of GAL4 from the membrane as a result of gamma secretase activity creates a cell death signal, and thus, flies with small eyes. This readout is very convenient for us because we can compare modifiers identified in GMR-rpr screens with those identified in GMR-APP-GAL4 screens. Those modifiers that are specific for GMR-APP-GAL4 are potentially interesting in terms of identifying genes that regulate APP cleavage. At this point we have carried out several large screen for enhancers and suppressors and have identified a modest number of interesting loci that are being pursued. Importantly, mutations that alter the levels of components of gamma secretase - presenilin and nicastrin - alter the reporter eye phenotype in the expected way. These observations give us confidence that the screen is likely to be pulling out interesting loci.

Doing a screen for modifiers of gamma secretase activity in a higher eukaryote is also important for the following reason. While gamma secretase is of course critical for cleavage of APP it is also likely to be important for the cleavage of other transmembrane signaling proteins such as Notch. Thus, drugs targeted directly at gamma secretase may have pleiotropic effects. A genetic approach that focuses more generally on identifying modifiers of this activity may point towards new ways of modifying its activity or specificity in ways that more specifically affect APP processing.

78. Gene activation screens for cell death regulators

Stephanie Vernooy, Soon Ji Yoo, Peizhang Xu, Cain H. Yam

The GMREP vector contains an eye-specific promoter near one P-element end, as well as sequences sufficient for plasmid rescue of genomic DNA flanking the site of P-element insertion. When this P element inserts near the 5' end of a gene it causes the gene to be misexpressed at high levels in the developing eye (Hay *et al*., 1997). We have mobilized this P element throughout the genome and are characterizing the insertions that act as cell death regulators. We first score the lines for dominant phenotypes that may be due to increased cell death (a small eye) or decreased cell death (a large, rough eye). We then cross these insertions to lines of flies that express the cell death activators REAPER, HID or GRIM specifically in the eye (GMR-rpr, GMR-hid, or GMR-grim flies), and that thus, have small eyes. The progeny of these crosses are then scored for the ability of the GMREP insertion to alter the GMR-*rpr*, GMR-*hid* or GMR-*grim*-dependent small eye phenotype. These modifiers identify new cell death regulators. Genomic DNA that is likely to contain a portion of the gene being overexpressed can be quickly isolated using plasmid rescue. The *Drosophila* genome is now finished. Thus, a small sequence tag from the end of the P element serves to tell us exactly where in the genome our insertion is. Because GMREP-dependent phenotypes are primarily due to insertions near the transcription start site, and because GMREP carries the dominant eye color marker *white*, imprecise P-element excision using a genomic source of transposase or X-rays can be carried out to rapidly generate deletions that create loss-of-function phenotypes for the overexpressed gene.

We generated and screened 7,000 transposon insertions for their ability to suppress *rpr*-, *hid*-, or grim-dependent cell death and identified a modest number of new loci (about ten) that specifically suppress death due to overexpression of one or the other, or all of these genes in the eye. We are in the process of characterizing some of these lines.

Reference

Hay, B.A., Maile, R. and Rubin, G.M. (1997) *Proc. Natl. Acad. Sci. USA* **94**:5195-5200.

79. IAPs, cell death and ubiquitination

Soon Ji Yoo, Jun Huh, Mark Zarnegar, H. Arno J. Muller

Viral and cellular IAPs which prevent cell death, as well as NAIP, contain N-terminal BIR repeats. Removal of the DIAP C-terminal RING finger does not eliminate death-preventing activity in response to *rpr* and *hid* overexpression in the *Drosophila* eye. These results suggest that the BIR repeats are sufficient to mediate DIAP-dependent death blocking activity. One goal of our work is to identify and characterize the domains of the DIAPs that are necessary for DIAP death-preventing activity in the fly. Several approaches are being taken toward this end. We are generating a large number of point mutants in specific, conserved residues within the BIR's, as well as carrying out truncations and domain swaps to ask if specific BIR's play distinct roles in death prevention.

Results from previous truncation and over-expression experiments suggest that the DIAP1 RING finger functions to negatively regulate or antagonize the BIR repeat-dependent death preventing activity. A second goal of our structure-function analysis is to determine the relationship between the DIAP BIR repeats and the RING finger by generating and testing the activity of a number of proteins that contain mutations in the RING domain, both in the context of the full-length protein, and in the context of the isolated RING domain.

DIAP1, as with many IAPs, also shows E3 ubiquitin-protein ligase activity. The function of this activity *in vivo* is unclear. One possibility is that this activity simply constitutes a mechanism for conferring a short half-life to the IAP, thus serving a proapoptotic function. Alternatively, ubiquitination of IAP-bound

proapoptotic proteins may provide a prosurvival mechanism by which IAPs can catalytically remove these molecules. IAPs may also engage in substrate choice, preferentially degrading themselves when not bound to proapoptotic molecules, but degrading binding partners when the opportunity arises. In this way IAPs could serve to create, through a post-transcriptional mechanism, a relative balance between pro-and antiapoptotic proteins, at different levels of proapoptotic proteins.

We, as well as several other labs, observed that expression of the death activators RPR, HID or GRIM led to a decrease in levels of the DIAP1 protein without a corresponding change in the levels of DIAP1 transcript. These observations suggested that DIAP1 protein levels were being regulated post-transcriptionally. We chased down two mechanisms by which this was being brought about, and by which it could regulate cell death. HID promotes the autoubiquitination of DIAP1 directly. In contrast, RPR and GRIM, while they promote the loss of DIAP1 protein, are able to do so even when DIAP1 has lost its ubiquitin protein ligase activity. This demonstrates that other processes must be at work. One likely possibility is that other E3s ubiquitinate DIAP1 in *trans*, thus promoting its degradation. However, in addition, we and the Kornbluth lab uncovered a second mechanism that is likely to be important - Rpr and Grim, but not Hid, promote a general suppression of protein translation.

This last observation is interesting because it has long been observed that inhibition of protein synthesis promotes cell death in many cell types. The observations from our lab and the Kornbluth lab can now provide at least a partial molecular explanation for this phenomenon: DIAP1 has a short half life, while that of its primary caspase target, Dronc, is much longer. Thus, when protein synthesis is inhibited an imbalance is created in the levels of DIAP1 with respect to its target caspase. From previous work we know that loss of DIAP1 is sufficient to promote cell death. Thus, all other things being equal, inhibition of protein synthesis should promote cell death.

A current focus of our work is to understand how RPR and GRIM inhibit protein synthesis and how HID promotes DIAP1 autoubiquitination**.**

Reference

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M.R., Clem, R.J., Muller, H.-A.J. and Hay, B.A. (2002) *Nature Cell Biol*. **4**:416-424.

80. Intracellular dynamics of the IAPs and caspases

Jun Huh, H-Arno J. Muller, Hong Yu, Lijuan Wang

We have antibodies that are specific for DIAP1, DIAP2, as well as a number of the caspases. We are using these reagents to determine the expression patterns of these proteins and their interrelationships throughout the fly life cycle. This analysis will tell us where these proteins are likely to be functioning to regulate cell death, and thus, where loss-of-function phenotypes may be apparent.

81. Loss-of-function phenotypes of the DIAPs

Bruce Hay, Jun Huh, H.-A. J. Müller

We are characterizing the loss-of-function phenotypes of DIAP1 and DIAP2. These phenotypes will identify DIAP gene functions that cannot be substituted for by the activity of other genes or developmental compensatory mechanisms. Homozygous DIAP1 mutant embryos arrest early during embryonic development, and essentially all cells appear to die. Clones of homozygous mutant cells are also not seen in other tissues. These observations all suggest that DIAP1 is required for cell survival, perhaps in all cells. We are characterizing these phenotypes in further detail, focusing particularly on the early embryonic phenotype. We have also generated double mutants that remove DIAP1 as well as genes contained within the 75C region (rpr, hid and grim). These mutants have a phenotype similar to that of the DIAP1 mutant alone, consistent with models in which *rpr*, *hid* and *grim* act through DIAP1 to induce cell death (**Figure 3**). Biochemical evidence supporting this model is presented below. We have also identified several P elements near the DIAP2 gene and are in the process of using these to generate DIAP2 mutants.

82. Identification of DIAP1-interacting proteins

Soon Ji Yoo, Hong Yu, Lijuan Wang

DIAP1 is required for cell survival in *Drosophila*. This suggests that its activity is likely to be regulated through interactions with other proteins. Genetic screens for cell death regulators provide one approach to identifying proteins that may interact with DIAP1. However, a more direct approach to identifying proteins that regulate DIAP1 function involves identifying proteins that physically bind DIAP1 in living *Drosophila*. We are using multiply tagged versions of DIAP1 as bait to immunoprecipitate and identify associated proteins from cells.

83. DRONC: An apical caspase required for RPR-, HID-, and GRIM-dependent cell death

Christine J. Hawkins, *Soon Ji Yoo*

One of our goals is to identify the caspase cascades used to promote *rpr*, *hid* and *grim*-dependent death. We (Hawkins *et al*., 2000; Meier *et al*., 2000) have found that the *Drosophila* caspase DRONC is critical for transducing these death signals because flies in which DRONC function is compromised show a dramatic suppression in RPR-, HID-, and GRIM-dependent death. DRONC also interacts with DIAP1 and HID and GRIM in *Drosophila*, in yeast and *in vitro* assays. The DRONC active site is very different from known caspases, suggesting that DRONC might have unique properties. We showed, using protein sequencing of DRONC substrates and *in vitro* mutagenesis that DRONC, unlike all other known caspases, processes itself following a glutamate. However, DRONC cleaves other caspases following an aspartate. DRONC activity is also not inhibited by the baculovirus p35 protein generally thought of as a pancaspase inhibitor. These observations, in conjunction with the results of a series of experiments in which we examined the ability of the four published *Drosophila* caspases to process each other, suggest a

model in which DRONC and only DRONC is able to process itself at a glutamate residue within the catalytic domain, generating an active caspase. This presumably serves to prevent DRONC activation by other caspases. Other caspases, however, are able to process the DRONC prodomain, providing a second level of regulation of DRONC activity. Once activated, DRONC rapidly cleaves downstream caspases, presumably triggering cell death.

Our observations with DRONC are important for several reasons. There are many situations in which caspase inhibitors are used to determine if particular cell deaths are apoptotic and caspase-independent. If the death is not suppressed by caspase inhibitors, it is usually argued that the death is caspase-independent. Our results suggest, however, that cell deaths that are clearly caspase-dependent (those mediated by *rpr*, *hid* and *grim*) involve a caspase-dependent step that has remained effectively invisible using inhibitors such as p35. It may well turn out to be the case that other pathological cell deaths that are thought to be caspase-independent (and thus, not amenable to caspase-based therapeutics), are in fact regulated by upstream proteases with nontraditional cleavage specificities.

References

Meier, P., Silke, J., Leevers, S.J. and Evan, G.I. (2000) *EMBO J.* **19**:598-611.

Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y. and Hay, B.A. (2000) *J. Biol. Chem.* **275**:27084-27093.

84. How does *Drosophila* activate caspase-dependent cell death?

Soon Ji Yoo, H.-A.J. Muller, Jun Huh

An important set of questions is how different cell death signals, which in many cases are initiated through distinct signal transduction pathways, converge to activate a set of common downstream effector pathways. In *Drosophila* many different death signals lead to the transcriptional activation of one or more of three genes, *reaper* (*rpr*), *hid* and *grim*. The function of these genes is required for most normally occuring and induced cell death in *Drosophila*. Thus, their transcriptional activation acts as a point of death signaling convergence. One of our primary goals has been to identify mechanisms by which these different proteins activate cell death. Three important facts are known about these proteins that suggested testable mechanisms of action. These are: 1) that death induced by their expression requires caspase activity; 2) that their death-promoting activity is suppressed in a dose-dependent manner by DIAP1; and 3) that REAPER (RPR), HID and GRIM bind to DIAP1 in insect cells (reviewed in Miller, 1999). These results have suggested several models of how DIAP1, caspases and RPR, HID and GRIM might interact to regulate cell death. In one model, death-activating proteins such as REAPER, HID or GRIM activate caspases through an IAP-independent pathway. This model postulates that *Drosophila* IAPs act at two different points to suppress apoptosis: by acting as a sink for death activators such as REAPER, HID or GRIM, preventing them from interacting with their normal targets, and by inhibiting the caspase activity initiated by their action. In a second model, DIAP1 is proposed to function primarily as a caspase inhibitor, and REAPER, HID and/or GRIM initiate caspase-dependent cell death by preventing IAPs from productively interacting with caspases, thereby promoting their activity, and ultimately cell death.

Susan Wang, a former graduate student, and Christine Hawkins, a former postdoc, carried out experiments in *Drosophila*, yeast and *in vitro* to test the idea that RPR, HID and GRIM promote apoptosis by blocking DIAP1's ability to inhibit caspase activity (Wang *et al*., 1999). They found that all three proteins, while nontoxic on their own, killed yeast coexpressing DCP-1 or drICE, and DIAP1, suggesting that they were blocking DIAP1's ability to function as a caspase inhibitor. They pursued the basis for this activity further with HID and found, both in yeast and *in vitro*, that proteins containing the N-terminal 37 residues of HID, which are sufficient to induce apoptosis in insect cells (Vucic *et al*., 1998), suppressed DIAP1's ability to inhibit DCP-1 activity.

These results are consistent with a model in which RPR, HID and GRIM act through DIAP1 to promote death-inducing caspase activity. This model predicts that DIAP1 should be essential for cell survival, and that a loss of DIAP1 function should result in an increase in DIAP1-inhibitable caspase activity. To test this idea they carried out a second set of experiments in which we characterized the phenotype of a DIAP1 loss-of-function mutation, as well as the phenotype of a double mutant that removed DIAP1, as well as *rpr*, *hid* and *grim*. They found that the DIAP1 loss-of-function phenotype consists of an embryo-wide set of cellular changes reminiscent of apoptotic cell death, and that these were associated with the activation of DIAP1-inhibitable caspase activity. Furthermore, double mutants that remove zygotic *rpr*, *hid*, *grim*, and DIAP1 function showed phenotypes similar to those of the DIAP1 loss-of-function mutant alone (Wang *et al*., 1999).

Together, the above observations suggest that a principal function of DIAP1 is to promote cell survival by blocking caspase activity, and that at least one mechanism by which REAPER, HID and GRIM promote apoptosis is by disrupting IAP-caspase interactions (**Figure 4**). These early studies left several important questions unanswered: 1) how does RPR, HID or GRIM binding to DIAP1 suppress DIAP1's ability to inhibit caspase activity; 2) do RPR, HID and GRIM regulate DIAP1 function through other mechanisms? Domain analysis of RPR and GRIM suggests that these proteins have apoptotic domains distinct from their N-terminal DIAP1 binding motifs. One question we are interested in is whether these other domains regulate DIAP1 through other mechanisms; 3) Finally, it is interesting to ask if there exist other proteins that function similarly to RPR, HID and GRIM. However, the yeast survival-based assay we used to show that these proteins disrupt IAP-caspase interactions provides a straightforward approach to screening for such molecules. Importantly, because such a screen is a function-based screen, and does not rely on identifying candidates based on sequence homology, we may identify proteins that disrupt IAP-caspase interactions even if they have only minimal homology to RPR, HID or GRIM.

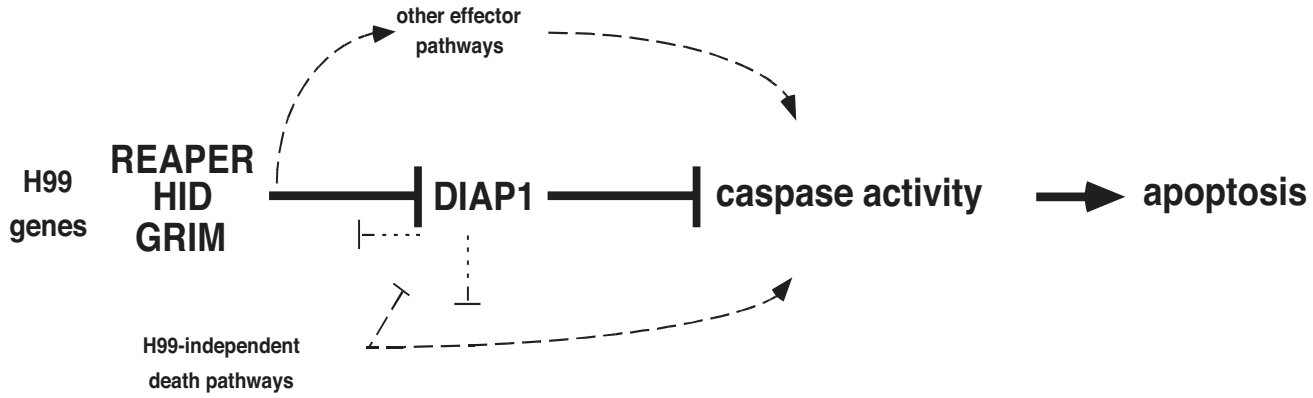

Figure 4. Model for how DIAP1 regulates apoptosis. DIAP1 inhibits caspase activity (⊣) and is essential for cell survival. DIAP1's ability to inhibit caspase activity is suppressed by RPR, HID and GRIM (⊣), thus, promoting caspase-dependent cell death (bold arrow). RPR and GRIM have other activities (Evans *et al*., 1997; Thress *et al*., 1998; Avdonin *et al*., 1999), suggesting that these proteins may also promote apoptosis through other pathways (dashed arrows). Free DIAP1 may inhibit apoptosis by binding these proteins, sequestering them from their targets (dashed-------|). Thus, DIAP1 may act both upstream and downstream of RPR, HID and GRIM to prevent cell death. Genes within the H99 interval are not required for some cell deaths (White *et al*., 1994; Foley and Cooley, 1998), indicating that other death pathways exist (dashed lines). It is not known if these pathways act through DIAP1.

References

White, K. *et al.* (1994) *Science* **264**:677-683.
Evans, E.K. *et al.* (1997) *EMBO J.* **16**:7372-7381.
Avdonin, V. *et al*. (1998) *Proc. Natl. Acad. Sci. USA* **95**:11703-11708.
Foley, K. and Cooley, L. (1998) *Development* **125**:1075-1082.
Thress, K. *et al.* (1998) *EMBO J.* **17**:6135-6143.
Vucic, D. *et al.* (1998) *Mol. Cell. Biol.* **18**:3300-3309.
Miller, L.K. (1999) *TICB* **9**:323-328.

85. Bruce, cell death, caspases and spermatogenesis

Stephanie Vernooy, Jun Huh

As mentioned above, one of the potent cell death suppressors we identified is the Bruce gene. Bruce mutants are viable, but they are male sterile. In examining this we discovered several interesting facts: 1) Bruce mutants are blocked in a late aspect of spermatogenesis known as individualization, in which spermatids (which develop in a common cytoplasm) eventually become enclosed in individual plasma membranes; 2) During the process of individualization spermatids have very high levels of active caspases. But these cells do not die. Together these observations suggest that spermatids use caspase activity for nonapoptotic purposes, during differentiation. Several questions are of interest to us: 1) What are the sources of the caspase activity (what are the upstream signals); 2) What are the nonapoptotic targets that facilitate differentiation; and 3) how is cell death prevented in the face of high levels of caspase activity that would normally be associated with cell death?

86. Autophagic cell death, caspase inhibition in *C. elegans*, and the *echinus* mutant

Jeffrey Copeland

While much cell death is apoptotic, a number of cell deaths share features with a process known as autophagy, which has been described in some molecular detail in yeast. In yeast, starvation leads to a cellular response in which double membrane-bound vesicles are formed that take up and hydrolyze organelles as well as bulk cytoplasm. This process of autodigestion provides the cell with nutrients, allowing survival under starvation conditions. It has been clear for some time that there are a number of situations in which cell death in animals shows morphological features similar to those of autophagy rather than apoptosis. However, the molecular mechanisms that mediate these deaths has remained unexplored. *Drosophila* homologs are available for many of the yeast proteins involved in autophagy. The goal of my project is to explore the molecular mechanisms underlying autophagic cell death in *Drosophila*.

In *C. elegans*, in contrast to the situation in flies and mammals, caspase inhibitors have not been identified. I used the yeast screens described above to identify several potential *C. elegans* caspase inhibitors. One of these is highly evolutionarily conserved. We are currently focusing our characterization on the *Drosophila* counterpart of this gene.

Echinus is a *Drosophila* mutant that lacks normally occurring cell death in the eye. I have generated new alleles of *echinus* and am characterizing genes in the surrounding genomic region.

Publications

Dorstyn, L., Read, S., Cakouros, D., Huh, J.R., Hay, B.A. and Kumar, S. (2002) The role of cytochrome *c* in caspase activation in *Drosophila* melanogaster cells. *J. Cell Biol*. **156**:1089-1098.

Guo, M. and Hay, B.A. (1999) Cell proliferation and apoptosis. *Curr. Opin. Cell Biol.* **11**:745-752.

Hawkins, C.J., Wang, S.L. and Hay, B.A. (1999) A cloning method to identify caspases and their regulators in yeast: Identification of *Drosophila* IAP1 as an inhibitor of the *Drosophila* caspase DCP-1. *Proc. Natl. Acad. Sci. USA* **96**:2885-2890.

Hawkins, C.J., Wang, S.L. and Hay, B.A. (2000) Monitoring the activity of caspases and their regulators in yeast. *Meth. Enzymol.* **322**:162-174.

Hawkins, C.J., Yoo, S.J., Peterson, E.P., Wang, S.L., Vernooy, S.Y. and Hay, B.A. (2000) The *Drosophila* caspase DRONC cleaves following glutamate or aspartate and is regulated by DIAP1, HID and GRIM. *J. Biol. Chem*. **275**:27084-27093.

Hay, B.A. (2000) Understanding IAP function and regulation: A view from *Drosophila*. *Cell Death Differ*. **7**:1045-1056.

Hay, B.A., Maile, R. and Rubin, G.M. (1997) P element insertion-dependent gene activation in the *Drosophila* eye. *Proc. Natl. Acad. Sci. USA* **94**:5195-5200.

Hay, B.A., Wassarman, D.A. and Rubin, G.M. (1995) *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**:1253-1262.

Hay, B.A., Wolff, T. and Rubin, G.M. (1994) Expression of baculovirus p35 prevents cell death in *Drosophila*. *Development* **120**:2121-2129.

Huh, J.R. and Hay, B.A. (2002) Sculptures of a fly's head. *Nature*. In press.

Sun-Yun Yu, Yoo, S.J., Yang, L., Zapata, C., Srinivasan, A., Hay, B.A. and Baker, N.E. (2002) A pathway of signals regulating effector and initiator caspases in the developing *Drosophila* eye. *Development* **129**:3269-3278.

Vernooy, S.Y., Chow, V., Su, J., Verbrugghe, K., Yang, J., Cole, S., Olson, M.R. and Hay, B.A. (2002) *Drosophila* Bruce can potently suppress Rpr- and Grim-, but not Hid-dependent cell death. *Curr. Biol*. **12**:1164-1168.

Vernooy, S.Y., Griffin, E.E., Ghaboosi, N., Copeland J. and Hay, B.A. (2000) Cell death in *Drosophila*: Conservation of mechanism and unique insights. *J. Cell Biol*. **150**:F69-F75.

Wang, S.L., Hawkins, C.J., Yoo, S.J., Muller, H.-A.J. and Hay, B.A. (1999) The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**:453-463.

Wu, J-W., Cocina, A.E., Chai, J., Hay, B.A. and Shi, Y. (2001) Structural analysis of a functional DIAP1 fragment bound to *grim* and *hid* peptides. *Mol. Cell* **8**:95-104.

Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M.R., Clem, R.J., Muller, H.-A. J. and Hay, B.A. (2002) Apoptosis inducers Hid, Rpr and Grim negatively regulate levels of the caspase inhibitor DIAP1 by distinct mechanisms. *Nature Cell Biol*. **4**:416-424.

Thomas Hunt Morgan Professor of Biology, Emeritus: Edward B. Lewis
Visiting Associate: Andrew Dowsett[1]
Postdoctoral Fellows: John Burr, Joanne Topol
Laboratory Staff: Lidia Bowman, Anna Campos, Martha Gomez, Blanca Granados, Jennifer Jeng, Aurora Marquina, Cory Olson, Mitzi Shpak
[1]*Claremont Colleges*

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Summary: Our first report involves a study of the *cis*-regulatory regions of the homeobox gene complex (HOX-C) of *Drosophila melanogaster.* The *cis*-regulatory regions of the HOX-C program virtually the entire course of the development of all higher animal organisms, including human beings, which have four semi-redundant sets of the complex. Our second report concerns a preliminary effort to try to identify new downstream targets of the HOX genes of the BX-C.

87. Are the major introns of the bithorax (BX-C) and Antennapedia complexes (ANT-C) interrelated to one another in DNA sequence?

E.B. Lewis

Drosophila is unusual in that the HOX-C is split into two separate complexes, the bithorax (BX-C) and Antennapedia (ANT-C) complexes. A unique opportunity exists in the case of the major HOX genes of the BX-C and ANT-C to test for relatedness in gene sequence among their introns. If those regions are ultimately derived from one another by tandem gene duplication as in the case of the homeobox portion of their coding regions, residual traces of that evolution might be expected to occur. More likely to be detectable, however, are sequences that are required for common *cis*-regulatory functions. Thus, since Ubx and Antp genes, e.g., have functions involving thoracic regions their introns might tend to be conserved for enhancer or other motifs, even though the genes must have diverged from one another over 100 million years ago. A Chi-squared method is being used to test for inter-relatedness in their intron sequences. The entire set of all possible hexanucleotides is compared for each of the possible pairs of major (over 15,000 base pairs) introns (after scaling to correct for size differences among the introns). The method suggests that the Ubx and Antp introns are more closely related to one another than they are to other introns of the BX-C and ANT-C. This result is consistent with their being closely related in function as well as being closely related by descent from a common ancestral HOX gene.

88. Functional analysis of the BX-C

John Burr, Cory Olson

The Berkeley *Drosophila* Genome Project is making considerable progress towards assembling a non-redundant set of cDNA clones from the ~14,000 genes believed to be present in *Drosophila melanogaster*[1,2]. In this report, we have used from that project cDNA clones for a set of transcription factors corresponding to ESTs (Expression Sequence Tags) in order to try to identify targets of the BXC transcription factors. A female fly carrying a transgene that contributes the Gal4 transcription factor to developing eggs in her ovarium[3] is mated with a male fly carrying a UAS^{GAL4}-*Ubx*, *abdominal-A* (*abd-A*), or *Abdominal-B* (*Abd-B*) cDNA[4]. The embryos derived from this cross ubiquitously overexpress the BXC homeotic gene at the onset of zygotic transcription. In a pilot screen, we generated anti-sense *in situ* RNA probes for each clone in a set of transcription factor cDNAs.

These probes detect where the transcription factor is expressed in the embryo. The expression pattern for each probe was assayed in wild-type embryos, and compared to embryos over-expressing a BXC homeotic gene. In the case of *Abd-B* over-expression, segmentation in the embryo is not disrupted, but all segments appear transformed toward an identity resembling the last two abdominal segments (where *AbdB* is endogenously expressed). Using this experimental design, we have identified the expected transcriptional silencing of *Ubx* and *abd-A* in response to ectopic *Abd-B* expression, as well as a few uncharacterized transcription factors that show changes in expression pattern.

Publications

Adams, M.D. *et al.* (2000) The Genome Sequence of *Drosophila melanogaster*. *Science* **287**:2185-2195.

Line NGT[11] is available from the Bloomington Stock Center, contributed by W.D. Tracey and J.P. Gergen.

Lines UAS-*Ubx*, UAS-*AbdA*, and UAS-*AbdB* are available from the Bloomington Stock Center, contributed by M. Akam.

Stapleton, M. *et al*. The *Drosophila* Gene Collection: Identification of putative full-length cDNAs for 70% of *D. melanogaster genes*. *Genomics Res*. In press.

George W. Beadle Professor of Biology: Elliot Meyerowitz
Postdoctoral Scholars: Marcio Ferreira, Venugopala Gonehal, Elizabeth Haswell, Marcus Heisler, Toshiro Ito, Jeffrey Long, Kazuaki Ohashi, Frank Wellmer, John Wyrick, Hao Yu, Yuanxiang Zhao, Eva Ziegelhoffer
Senior Research Fellow: Carolyn Ohno
Visiting Associates: James P. Folsom, Jose Luis Riechmann
Graduate Student: Catherine Baker
Undergraduate Students: Jennifer Li, Anongpat Suttangkakul, Stephan Wenkel
Research Laboratory Staff: Leonard Medrano, Maral Robinson

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Summary: We are trying to understand how plants develop, with particular emphasis on the processes of organ specification in flower development, and of cell-cell communication and cell division control in shoot apical meristems, the growing tips of each shoot.

Earlier work in this lab and others indicates that a small set of regulatory genes called the ABC genes controls the nature of the floral organs to which organ primordia will develop, with various combinations of the genes promoting the development of the five possible organ types in flowers: leaves, sepals, petals, stamens, and carpels. Most of the ABC genes code for transcription factors in the MADS-domain family, a type of DNA-binding protein found in fungi and animals, but with a particularly large number of different family members serving key developmental functions in plants. One open question is the identity and nature of the genes activated or repressed by the ABC regulatory genes during flower development. To find out we have designed a number of plant genotypes in which different of the ABC genes can experimentally activated. We are using DNA probes made from RNAs extracted from these plants at different time points after activation, on microarrays representing flower-specific *Arabidopsis* genes. The results are providing a detailed list of the gene activities that depend on ABC regulation, and therefore a view of the mechanism by which these genes regulate floral organ identity. Another open question concerns the positions in which new floral organs or leaves appear, regularly, in developing plants. New experiments with the plant hormone auxin are providing a promising route to understanding this long-standing problem in plant development.

One new element in our work on shoot apical meristems has been the development of new microscopic methods to observe populations of cells in living meristems over a period of several days. Such observations are leading to a new level of understanding of the behavior of cells in shoot tips, and of their gene expression patterns. We also continue to make progress in cloning and characterizing the function of new genes that affect flower and shoot development, such as *HANABA TARANU*, coding for a GATA-box transcription factor, *TOPLESS*, encoding a WD40 protein and *SHREDDIE*, producing a zinc-finger protein. More on each of these genes, and others, is in the abstracts that follow.

89. Gene expression analysis of *Arabidopsis* flower development

Frank Wellmer, Jose Luis Riechmann, Marcio Alves-Ferreira

For a better understanding of *Arabidopsis* flower development we are currently generating an expression map for genes that are expressed only at certain time points during flower development, or specifically in certain parts of the flower. These spatially and/or temporally regulated genes may play an important role in the regulatory processes that pattern the flower or in the differentiation of the various types of floral tissues. In order to study gene expression on a genomic level we have constructed an 10,800 element cDNA microarray that is composed mainly of flower-specific transcripts.

We have initiated our study by comparing the gene expression patterns of wild-type flowers with those of mutants that show homeotic transformations. In these mutants certain types of floral organs are absent or are replaced by other types of organs. By combining the data sets obtained in these experiments we were able to identify groups of genes that are predicted to be specifically expressed or strongly enriched in one of the four different floral organs: sepals, petals, stamens and carpels. The predicted expression patterns of several of the presumably organ-specific genes were confirmed by *in situ* hybridizations. Furthermore, the analysis of several genes with known organ-specific expression showed that their expression patterns were predicted correctly by the microarray experiments.

We are now completing our study by determining the temporal expression of genes during flower development. For this, we are comparing the expression patterns in floral buds of different stages. The analysis of the spatial and temporal expression of genes during flower development does not only reveal interesting genes with possible regulatory functions, but also provide a substantial set of marker genes that can be used for the detailed analysis of flower development.

90. Identification of target genes of MADS transcription factors involved in *Arabidopsis* flower development

Frank Wellmer, Jose Luis Riechmann, Toshiro Ito, Marcio Alves-Ferreira

During flower development the floral meristem is partitioned into four concentric whorls that give rise to the different types of floral organs. The specification of floral organs is mainly regulated by transcription factors that contain the so-called MADS-box domain, namely: APETALA1; APETALA3; PISTILLATA; and AGAMOUS, as well as three functionally redundant SEPALLATA proteins. Different combinations of these factors are required for the formation of a certain type of floral organ. For example, stamen fate requires the activity of APETALA3, PISTILLATA, AGAMOUS and SEPALLATA proteins, whereas AGAMOUS and SEPALLATA proteins alone are sufficient for the formation of carpels. Furthermore, it has been shown that some of these factors interact physically. The factors might therefore form regulatory complexes that control genes involved in organ formation.

Despite our increasing knowledge of the regulatory mechanisms that are involved in flower development it is unclear which genes are regulated by the MADS-box factors. We are currently trying to identify the target genes of these transcription factors using microarrays. For this, we have fused the coding regions of the factors with a fragment of the glucocorticoid receptor and express the resulting fusion proteins in plants. These fusion proteins can be specifically activated by treating the transgenic plants with a steroid hormone. This system allows us to do time course experiments and observe changes in gene expression that occur shortly after the activation of the transcription factors as well as later changes that are presumably downstream of the primary events.

The activity of AGAMOUS-GR, for example, can be studied in flowers of *agamous* mutants. (The activation of AGAMOUS-GR rescues the mutant phenotype; see Toshiro Ito's abstract in this section for a more detailed description.) Since AGAMOUS is involved in the specification of stamens as well as carpels, it would not be immediately clear to which type of organ certain target genes contribute. This problem could be addressed by an alternatively approach in which AGAMOUS-GR and SEPELLATA-GR are co-expressed in leaves. The simultaneous activation of both fusion proteins would then start the developmental program for carpels but not for stamens (see above) allowing a clear distinction between organ specific target genes of AGAMOUS.

This work has been done in collaboration with Pedro Robles and Martin Yanofsky of UC San Diego.

91. Searching for the AGAMOUS target genes based on microarray screening

Toshiro Ito, Frank Wellmer, Jose-Luis Riechmann

The loss of function of the floral homeotic gene *AGAMOUS* (*AG*), which encodes a member of the MADS box family of transcription factors, results in flowers with floral organ identities (from whorl 1 inward) of sepal-petal-petal repeating indefinitely. Downstream cascades of AG that lead to stamen and carpel morphogenesis is a long-time mystery, and one of our research aims is to reveal the downstream activities of AG.

We have been doing the high-throughput flower cDNA microarray screening using transgenic plants with an inducible AG activity. A strain was created that was homozygous for *ag-1* and transgenic for the construct 35S::*AG-GR*, that is, virus 35S protein promoter-driven *AG* gene with a carboxyl-terminal fusion to the steroid binding domain of the rat glucocorticoid receptor. When untreated, plants of this genotype make *ag-1* mutant flowers, with successive whorls of sepal-petal-petal indefinitely. Treatment with dexamethasone (DEX) leads to flowers with (from whorl 1 inward) carpelloid sepal-stamen-stamen-carpel like 35S::*AG* flowers.

As a time course experiment after induction of AG activity, inflorescences from 35S::*AG-GR ag-1* mutant plants were collected at 0, 4, 8, 10, 12 and 16 hours after mock- or DEX-treatment, and RNA was isolated from each time sample. Three sets of independent RNA samples from 35S::*AG-GR ag-1* plants and two sets of RNA samples from *ag-1* single mutants were isolated. These RNA samples were used to prepare Cy3- and Cy5-labeled probes for cDNA microarray hybridization, allowing us to compare the RNA expression profiles of mock- and DEX-treated 35S::*AG-GR ag-1* plants at the each time point. We used RNA samples from *ag-1* single mutant plants to exclude genes that were induced by the dexamethasone treatment itself. Once candidate target genes are identified, we will determine if they are induced in the presence of cycloheximide, an inhibitor of protein synthesis, in order to identify genes that are directly regulated by the AG protein. Repeated hybridization and confirmation of data sets are under way.

92. Developing bioinformatic tools to investigate *Arabidopsis* genome expression

John J. Wyrick

The recent completion of the *Arabidopsis* genome sequence and the development of DNA microarray technology have opened new avenues to investigate the global regulation of plant gene expression during development. To facilitate these investigations, a variety of computational tools are needed to analyze and interpret these data. We have developed a relational SQL database, named AtchipDB, to store and analyze the gene expression data generated by *Arabidopsis* DNA microarrays. The database is web-accessible and provides a suite of tools to analyze and display the data stored within. We are in the process of implementing tools in AtchipDB that will allow the user to cluster microarray data and to search for

conserved DNA sequence motifs in the promoter regions of coregulated genes.

93. Bioinformatics approach searching for the AGAMOUS target genes

Toshiro Ito

We also have been adopting bioinformatics approach in order to identify direct target genes regulated by the floral homeotic protein AGAMOUS (AG).

AG protein forms a homodimer *in vitro* and shows specific binding to a relatively long sequence, 5'-TT(A/T/G)CC(A/T)6GG(A/T/C)AA-3'CC(A/T)6GG. Using the NCGR PatMatching database search program against the entire genome sequence of *Arabidopsis*, we previously found 1,007 potential AG binding sequences. By searching the nearby regions of these sequences, we found 24 genes that were expressed in wild-type flowers, and that showed either no expression, or highly reduced expression, in *ag-1* mutant flowers, indicating at least that they are predominantly expressed in developing stamens and carpels, the domain of *AG* expression.

A direct test of AG inducibility was then performed using plants transgenic for 35S::AG-GR in the *ag-1* mutant background. When untreated, plants of this genotype make *ag-1* mutant flowers. Continuous treatment with dexamethasone (DEX) leads to flowers with normal stamens and carpels in whorl 3 and whorl 4, respectively and flowers which finally produce fertile seeds from *ag-1* homozygous background. By using this strain, the expression levels of each candidate gene were tested by RT-PCR in the floral buds after AG induction with or without the translation inhibitor cycloheximide. So far three genes appear to be direct targets of AG (induced in DEX and in DEX + translation inhibitor cycloheximide). One is a member of the WRKY-type zinc finger transcription factor family, one a member of the homeobox transcription factor family and one a member of the AT-hook DNA binding protein family. We also identified early induced, but not direct targets of AG (induced in DEX after 6 hours, but not induced in DEX + cycloheximide). One is a cell-wall disconnecting protein and two are members of the C2H2-type transcription factor family. *In situ* hybridization, overexpression in wild-type and *ag-1* mutant background, and the production of knockout and RNAi mutations for these genes are in progress.

94. Functional analysis of a direct target of AG, *AGT2ATH*

Toshiro Ito

Based on a bioinformatics approach, we identified a member of the AT hook-type DNA binding protein family (AGT2ATH) as one of direct target genes of AGAMOUS (AG). The *AGT2ATH* gene was expressed in roots and flowers, but not in leaves, as shown by RT-PCR. Next, we examined the expression of *AGT2ATH* in flowers by *in situ* hybridization experiments. The *AGT2ATH* gene was predominantly expressed in whorl 3 and whorl 4 of floral primordia at stage 3 afterward, the domain of AG expression. *AGT2ATH* is also expressed in emerging ovule primordia. The overall expression patterns of *AGT2ATH* matched with that of *AG*, suggesting that AGT2ATH may be involved in the morphogenesis of both stamens and carpels as a direct target of AG in flower development.

In order to examine the developmental function of the *AGT2ATH* gene, we ectopically induced *AGT2ATH* activity by a transgenic construct of 35S::*AGT2ATH*. As a result, 35S::*AGT2ATH* plant showed a characteristic vegetative phenotype of curled-up leaves, which was also observed in 35S::*AG* plants. In the flowers of 35S::*AGT2ATH* plants, the petals were curled inward at the top, but no homeotic conversion was observed. In 35S::*AG* flower, organs in whorl 2 (petals in wild type) are transformed into stamens, which curl inward at the top, suggesting that AGT2ATH may not have organ identity functions, but may be involved in cell division or cell elongation during stamen and carpel morphogenesis.

We also identified a knockout mutant of *AGT2ATH* gene from T-DNA insertional pools of UWBC by PCR-based screening. The T-DNA was inserted right at the initiation methionine site and appears to disrupt the initiation codon of *AGT2ATH*. By RT-PCR under regular conditions, no full-length cDNA was detected in the homozygote for the T-DNA insertion. The insertional mutant plant did not show any drastic homeotic or morphological alterations, but showed pale-colored carpels. Detailed analysis of surface structure of the carpels cell is under way. The subtle phenotype of *agt2ath* mutant may be due to the redundancy of other members of AT-hook DNA binding protein family, and so we are also searching for insertional lines of two highly similar members of the AT-hook DNA binding protein, aiming to produce a double or triple mutant plant.

95. Analysis of SUPERMAN functions in floral meristem development

Toshiro Ito

The *superman* (*sup*) mutant has extra whorls of stamens and underdeveloped carpels. A double-mutant of *sup* and *agamous* results in a very large flower consisting of repetitive petals with a prolonged and fasciated meristem in the center. In order to investigate how SUP affects floral meristem determinacy, we previously performed an ectopic expression analysis of SUP by a transgene of 35S::*SUP-GR*, that is a translational fusion between SUP and the hormone binding domain of a glucocorticoid receptor (GR). Ectopic SUP activity caused the inflorescence meristem to be terminated with carpelloid organs and the flowers to become very small, with four carpels. In extreme cases, by a strong continuous DEX treatment, the flowers were transformed into leaves. These results suggest that ectopic SUP activity can promote the differentiation of inflorescence meristems and floral meristems, and terminate meristematic activity.

This hypothesis is further supported from the analysis of transgenic plants for *SUP* promoter-driven

SUP-GR in the *sup-5 ag-1* double mutant background. By continuous DEX treatment of *pSUP::SUP-GR*; *sup-5; ag-1* inflorescences, later-arising flowers show a single *ag-1* mutant phenotype. Interestingly, however, the early arising flowers (having experienced a short period of SUP activity) showed multiple carpelloid organs in the peripheral region of a less-fasciated meristem, suggesting that the SUP-GR fusion protein driven by a *SUP* promoter also induces the differentiation of organs from the peripheral region of the meristems. From these observations, we propose that, in addition to the previously published model that shows SUP works as a boundary to prevent the proliferation of the whorl 3, SUP has an additional activity that promotes the differentiation of the meristem into floral primordia. Molecular analysis of this determinacy control by SUP is being done by microarray analysis.

96. Searching for the SUPERMAN target genes by microarray experiments

Toshiro Ito, Jose-Luis Riechmann, Frank Wellmer, Marcio Alves Ferreira

The *SUPERMAN* (*SUP*) gene, which encodes a C2H2-type zinc-finger transcription factor, has a determinacy function in the center of the floral meristem. In order to reveal the molecular nature of the downstream activities of SUP in floral meristem development, we produced an inducible system of SUP activity and searched for induced/repressed genes by using flower cDNA microarrays. In order to get larger amounts of material, and enhanced effects of the transgene, we introduced a 35S::*SUP-GR* construct into a severe *clavata* mutant, *clv3-2*. The 35S::*SUP-GR clv3-2* mutant flowers showed extra whorls of carpelloid organs in the center upon DEX induction. As a time course experiment after induction of SUP activity inflorescences from 35S::*SUP*-GR *clv3-2* mutant plants were collected at 0, 4, 8, 12 and 24 hours after mock- or DEX-treatment, and three sets of RNA were isolated from each time sample. As a result of microarray hybridization, we found several genes that were repressed just 4 hours after SUP induction, but no induced genes were found, suggesting that SUP functions as a repressor for transcription. One is *INNER NO OUTER* (*INO*) gene. *INO* encodes a member of the YABBY family of putative transcription factors and is expressed in the presumptive outer integument on the abaxial side of ovule primordium. Previous work showed that the *ino* mutants were epistatic to *sup* in ovule development, and that in *sup-5* ovules, *INO* was ectopically expressed in both sides of the outer integument and also in the funiculus (Gaiser *et al*., 1995; Villenueva *et al*., 1999). According to our RT-PCR results using 35S::*SUP-GR* plants, the expression of *INO* is repressed in DEX and also in DEX with a protein synthesis inhibitor, cycloheximide, strongly suggesting that SUP directly suppresses *INO* expression.

We also identified *Wall Associated Protein Kinase 2* as a direct target of SUP. Cell-Wall Associated protein Kinases (WAKs) are one of the protein families that physically link the extracellular matrix (ECM) to the plasma membrane and may facilitate communication between the ECM and cytoplasm (Wagner and Kohorn, 2001). Previous work showed that *WAK2* is expressed in floral buds, in addition to strong expression in seedlings, shoot apical meristems and young leaves, and that antisense expression of *WAK2* inhibits cell elongation and reduces plant size. Our work showed that ectopic SUP expression reduces the plant and flower size by inhibiting the cell elongation. The repression of *WAK2* gene by SUP may be one of the causes of this dwarfness. Detailed expression and functional analysis of these target genes of SUP are currently under way.

References

Gaiser, J.C., Robinson-Beers. K. and Gasser, C.S. (1995) The *Arabidopsis* SUPERMAN gene mediates asymmetric growth of the outer integument of ovules. *Plant Cell* **7**:333-345.

Villanueva, J.M., Broadhvest, J., Hauser, B.A., Meister, R.J., Schneitz, K. and Gasser, C.S. (1999) *Genes Dev.* **3**:160-169.

Wagner, T.A. and Kohorn, B.D. (2001) *Plant Cell* **13**:303-318.

97. Characterization of *AGL24* in the control of flowering time

Hao Yu

This flowering process is regulated by several interacting pathways that monitor both the developmental state of the plants and environmental cues such as light and temperature. The flowering-time genes *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), together with the floral meristem identity gene *LEAFY* (*LFY*), are three essential regulators integrating floral signals from multiple pathways in *Arabidopsis thaliana*. Our previous studies showed that part of the crosstalk among these genes is mediated by a putative transcription factor, *AGAMOUS-LIKE 24* (*AGL24*). This gene is gradually activated in shoot apical meristems during floral transition and later located in the whole zone of both inflorescence and floral meristems. Loss and reduction of *AGL24* activity by double-stranded RNA (dsRNA)-mediated interference result in late flowering, while constitutive overexpression of *AGL24* causes precocious flowering. The correlation between the level of *AGL24* accumulation and the alteration of flowering time suggests that *AGL24* is a dosage-dependent flowering promoter.

The detailed studies of *AGL24* expression in various flowering-time mutants show that it is regulated in several floral inductive pathways including the photoperiod-, vernalization-, GA-, and autonomous-promotion pathways. Further genetic analyses of epistasis describe the scenario in which *AGL24* acts downstream of *SOC1* and upstream of *LFY*. Our current work is focused on the identification of putative target genes of *AGL24*. We are using the inducible AGL24-GR system and activation tagging to isolate downstream genes or related enhancers and suppressors. Since *AGL24* acts upstream of

LFY, this series of studies will clarify if *AGL24* is a direct upstream gene of *LFY*, and if not, which are other intermediates mediating the flowering signals from upstream pathways to *LFY*. The eventual finding of certain direct upstream genes of *LFY* will provide insight into the mechanisms by which LFY plays a significant role, not only in flowering time control, but also in floral organ formation.

98. Genome-wide binding site analysis of floral regulators by chromatin immunoprecipitation in *Arabidopsis*

Toshiro Ito

In order to identify target genes that are directly controlled by floral regulators, we started a chromatin immunoprecipitation (ChIP) analysis of genome-wide binding sites using three different classes of floral regulators, LEAFY (LFY), AGAMOUS (AG) and SUPERMAN(SUP). Although this method has been successfully applied to a single-celled eukaryote (*S. cerevisiae*), it has technical limitations of its sensitivity in multicellular organisms. In order to overcome the limits of the application of ChIP to a multicellular plant system, we improved the previous method in three ways: 1) to obtain homogeneous populations of cells with inducible gene activity; 2) to use epitope tags; and 3) to use an *Arabidopsis* DNA microarray.

First, we made vectors in which the GR domain is followed by a carboxyl-terminal epitope tag (HA or c-myc). In addition the vectors have double 35S enhancers with a core promoter, followed by multiple cloning sites for cDNAs, and then a GR-epitope tag. Second, we cloned *AG* (in combination with *SEP3*), *LFY* and *SUP* cDNAs into the vectors and transformed these constructs into *Arabidopsis* wild-type plants. The AG-SEP complex was shown to be sufficient to induce carpels. We also produced transgenes expressing SEP3, AP3 and PI in addition to AG to have cells forming stamens. We were reasonably convinced that such callus cultures would be useful from experiments in which we had activated 35S::*LFY-GR* in similar cultures, leading to simultaneous formation of flowers all over the callus tissue (Wagner, Kumar and Meyerowitz, unpublished).

As a result of multiple transformations, a total of 289 T1 transformants were obtained. Next, proteins were isolated from the cauline leaves of these T1 transformants, and GR-epitope fusion proteins with AG, LFY or SUP were immunodetected by Western blots using epitope-antibody. Out of 289, 48 % (140 lines) showed detectable expression and of these 21% (30 lines) showed strong expression. From these lines with strong expression, we started to produce callus by putting the inflorescences on the callus-inducing medium. When we obtain enough callus tissue, we will start the expression test by PCR using primers for known target genes (*AGT2ATH* and *AGT4HOX* for AG; *AP1* and *SEP2* for LFY; *INO* and *WAK2* for SUP), and then chromatin immunoprecipitation experiments to detect the direct binding sites of AG, LFY and SUP *in vivo*.

99. Effect of ectopic expression of WUSCHEL function

Yuanxiang Zhao

WUSCHEL (*WUS*) encodes a homeodomain protein that is involved in regulating meristem size control in *Arabidopsis*. It is expressed in the subepidermal cells beginning in the 16-cell embryo, and is later confined to the central cells of the L3 layer in the shoot apical meristem (SAM) but excluded from the L1 and L2 layers of cells where CLAVATA3 (CLV3) is expressed (Mayer *et al*., 1998). CLV3 encodes a secreted signal peptide and binds to its receptor CLAVATA1 (CLV1) which is expressed in the rib meristem zone of SAM embracing the WUS expression domain (Fletcher and Meyerowitz, 2000). A regulatory loop between WUS and CLV3-CLV1 pathways has been shown to be essential for SAM maintenance. WUS expression promotes stem cell proliferation and meristem growth. This results in increased CLV3 expression, which in return negatively regulates WUS expression to restrict meristem size. As WUS is expressed very early on during embryo development, it might also play important roles in establishing the SAM structure. To test that, we will ectopically express WUS under the control of the MERISTEM LAYER 1 (ATML1) promoter. This promoter has been shown to confer high-level expression in the epidermis of developing embryos beginning from 16-cell stage embryos and the L1 layer of shoot and floral meristems, where WUS is normally absent. Plants carrying the pAtML1::WUS transgene will be examined, and the SAM development and organization will be followed by checking the CLV3, CLV1 and STM expression. Two different approaches have been taken to ectopically express WUS under the AtML1 promoter. First, a LHG4;6XOP two component system was utilized. The activator line is pAtML1::LHG4 and the reporter line is 6XOP::WUS. Upon crossing between these two lines, WUS will be activated. The second approach is to make use of an inducible system. A fusion protein, WUS-GR (glucocorticoid receptor steroid binding domain), was expressed under direct control of the AtML1 promoter. The translational fusion protein will remain in the cytoplasm until dexamethasone application, which enables nuclear transport. Both systems will avoid the possibility of inability to obtain the pAtML1::WUS mutant lines due to embryo lethality. Currently the T1 generation transformants have been selected and positive transgenic lines obtained.

References

Mayer, K.F.X., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998) *Cell* **95**:805-815.

Fletcher, J.C. and Meyerowitz, E.M. (2000) *Curr. Opin. Plant Biol*. **3**:23-30.

100. Functional analysis of GATA-3 like transcription factors in *Arabidopsis*

Yuanxiang Zhao, Kazuaki Ohashi, Leonard J. Medrano

The GATA factors are a family of transcriptional regulatory proteins characterized by the ability to bind the consensus DNA sequence (T/A)GATA(A/G), through a highly conserved DNA-binding domain comprised of one or two type-IV zinc finger motifs (C-x2-C-x_{17-18}C-x2-C). They have been found in fungi, *Drosophila*, *C. elegans*, *Xenopus*, mouse, humans and also plants. While both single and two zinc finger motif-containing GATA factors have been isolated in other organisms, only single zinc finger GATA factors have been isolated or predicted from the genome sequence data in plants. In addition, animal GATA factors only contain the C-x2-C-x17-C-x2-C-type zinc finger motif, and fungi have both the C-x2-C-x17-C-x2-C and C-x2-C-x18-C-x2-C configurations, whereas plants have only the latter. Although GATA factors have been extensively characterized in animals and fungi, their roles in plants are still waiting to be discovered. The *Arabidopsis* genome sequence database reveals 28 potential GATA-like transcription factors. One mutant, *hanaba taranu* (*han*) affecting flower development was isolated through an Agrobacterium-mediated T-DNA mutagenesis. Normal flowers contain four sepals, four petals, six stamens and two carpels fused into one. The *han* mutant flower has fused sepals (2-3 numbers), complete loss or decreased numbers of petals and stamens, and sometimes unfused carpels. The gene turned out to encode a GATA-like factor. Database searches revealed two homologues in *Arabidopsis* (HANL1 and HANL2) that share 40% and 44% amino acid identity with HAN. Mutational lines for the two homologues were isolated through PCR-based screening of T-DNA insertion lines and their phenotypes are being analyzed. We are in the process of characterizing the expression pattern of these three genes and generating double and triple mutants for functional comparison.

101. The *SHREDDIE* (*SDI*) locus encodes a C_2H_2 zinc finger protein that functions in growth of floral organs

Carolyn Ohno, G. Venugopala Reddy

Mutations in the *SDI* gene cause narrow floral organs, giving the flower a shredded appearance. The *SDI* gene has been identified by a map-based cloning strategy after analysis of a F2 mapping population containing 428 mutant plants that were generated from a cross between the *sdi* mutant (Landsberg *erecta ecotype)* and wild-type Columbia ecotype. The *SDI* locus is found to encode a C_2H_2 zinc finger protein. Other notable protein motifs include a proline-rich motif at the carboxyl-terminal end and a transcriptional repressor motif in the amino-terminal end of the coding sequence. The *sdi* mutant phenotype has been complemented with two independent genomic fragments that encompass the *SDI* locus but are not predicted to contain additional loci. Sequencing of the *sdi-1* allele revealed a G to A transition at the 3' splice site of intron 4. The second EMS induced lesion in the *sdi-2* allele is a C to T transition resulting in a premature stop codon within the zinc finger domain.

In situ hybridization experiments revealed a cell-specific developmental pattern of *SDI* expression that is associated with developing organ primordia, while meristematic tissues showed no detectable expression. *SDI* mRNA is detected in the emerging cotyledons of the transition-stage embryo, in vegetative leaf primordia and in developing floral organ primordia. In the inflorescence *SDI* transcripts are first detected in stage 2 to 3 flowers in the cells associated with the sepal primordia that are the first floral organ primordia to develop. In stage 4 to 8 flowers expression is associated with emerging petal, stamen and carpel primordia, while expression in sepals decreases. Within the gynoecium, *SDI* is expressed in the carpel valve and in ovules. Preliminary scanning electron microscope analyses have revealed that both cell expansion and cell shape are altered in the narrow organs of the *sdi* mutant flowers. Thus, the *SDI* gene may play a role in transcriptional regulation of cell expansion and/or division in reproductive as well as vegetative organ primordia. Studies of *SDI* misexpression in transgenic plants will further reveal the developmental function *SDI* plays in organ morphology.

102. TPL represses root face in the embryonic shoot

Jeff A. Long

Arabidopsis embryos develop with a distinct apical/basal polarity. Two groups of stem cells, the shoot apical meristem and the root apical meristem, form at opposite poles of the embryo (the apical and basal poles respectively). *topless-1* (*tpl-1*) is a single, temperature-sensitive mutation that transforms the shoot pole of the embryo into a second root pole. *tpl-1*, which was originally identified by Dr. Kathryn Barton, is the only *Arabidopsis* mutation identified to date that can cause homeotic transformation of the embryo.

TPL encodes an 1131 amino acid protein containing 12 predicted WD40 repeats and a proline-rich domain. These domains are also found in the TUP1/GROUCHO family of transcriptional corepressors. Four other predicted proteins that share at least 74% amino acid similarity with TPL are found in the *Arabidopsis* genome.

In situ hybridizations show that *TPL* mRNA is broadly expressed during embryogenesis with the highest levels found in the embryo proper and in the developing vasculature. A TPL-GFP translational fusion protein localizes to the nucleus in transgenic plants and can rescue the *tpl-1* mutation when placed under the control of the putative *TPL* promoter, indicating that TPL is likely to encode a nuclear protein.

In an EMS mutagenesis, we isolated three strong suppressors of *tpl-1*. All 3 of these suppressors are second site mutations in the *TPL* gene and are predicted to disrupt gene function. This shows that *tpl-1* is a gain of function allele. When these intragenic suppressors are made homozygous, they appear to be wild type, suggesting that

another gene or genes are functionally redundant with *TPL*.

In order to overcome the apparent redundancy of the *TPL* gene family, we built an RNAi construct that should downregulate several family members. When this construct is expressed in flowers, we find that most floral organs are transformed into stamens. This phenotype is reminiscent of what we have observed with the *tpl-1* allele at high temperatures.

Taken together, these data have lead us to a model wherein TPL acts as a transcriptional corepressor and is required to prevent root-promoting genes from being expressed in the shoot. The original *tpl-1* allele appears to be a dominant negative for several TPL family members and gives us a unique tool to investigate how embryos determine and then maintain their apical/basal polarity.

103. *AtGCN5* is necessary for shoot to root transformations in *topless-1* mutant embryos

Jeff A. Long

In order to gain insight into the function of the TOPLESS (TPL) protein, we took advantage of the temperature sensitivity of the *tpl-1* allele. At the restrictive temperature, the majority of *tpl-1* embryos display a transformation of the shoot into a second root. We mutagenized *tpl-1* homozygous seeds with both EMS and gamma rays and allowed the plants to set seed at the restrictive temperature. We then screened the resulting population for plants that had a wild-type appearance.

In this screen, one strong, recessive suppressor was isolated and named *big top* (*bgt)*. *bgt* plants carry a mutation in the *Arabidopsis* homologue of the yeast GCN5 protein, a histone acetyltransferase. In yeast, GCN5 is recruited to chromatin by DNA binding transcription factors, where it can act as a transcriptional coactivator. This result indicates that genes necessary to form the apical root in *tpl-1* embryos are under the control of AtGCN5 and implicates chromatin remodeling in embryonic polarity decisions. This result also further supports our model for TPL as a transcriptional corepressor.

Plants homozygous for *bgt* display a variety of developmental defects not associated with *TPL* gene function. *bgt* plants are small, have serrated leaves and are partially male and female sterile. This suggests, that like in yeast and other organisms, AtGCN5 is involved in the regulation of multiple genes. By using inducible versions of *TPL* and *BGT*, coupled with microarray analysis, we hope to identify the subset of genes that are regulated by both proteins.

104. *early extra petals* (*eep*) affects petal number in *Arabidopsis thaliana*

Catherine Baker, Frank Wellmer

In wild-type *Arabidopsis thaliana*, floral organ numbers are largely invariant, with each flower containing four sepals, four petals, six stamens, and two carpels. Various mutations that increase the size of the floral meristem (*clavata1*, *clavata3*, *wiggum*, *ultrapetala*) or cause the floral meristem to be smaller than wild type (*wuschel*) show an increase or decrease, respectively, in the number of organs in two or more whorls. Floral organ numbers can vary, however, in the absence of a dramatic defect in meristem size, as demonstrated by the *perianthia*, *pinoid* and *pinformed* mutations. Plants homozygous for these mutations have an increased number of petals, and the latter two mutants show additional phenotypes associated with disruptions of auxin signaling and/or transport.

In the interest of further uncovering what governs the number and position of floral organs within a whorl, we are studying a mutant called *early extra petals* (*eep*). Both *eep -1* and *eep-2* plants have extra petals in the first 5-10 flowers, without affecting the organ numbers in other whorls (unlike *clv1*, *clv3*, *wig*, *ult*, and *pan*). In addition, they lack the pleiotropic auxin-related phenotypes associated with *pinoid* and *pinformed*.

Using a map-based approach, we have localized the *eep* mutation to a 50-kb region on chromosome 5. No mutation has been identified in the predicted open reading frames within this region; we are transforming fragments of genomic DNA from this region in order to rescue the *eep* phenotype. In addition, we are analyzing *eep* double mutants with other mutations known to alter petal number and/or organ identity.

105. Live imaging of meristems

G. Venugopala Reddy, Marcus Heisler

The shoot and its attendant structures such as leaves and flowers develop from undifferentiated proliferative zones called shoot apical meristems (SAMs). The SAM is sub-divided into central zone (CZ), peripheral zone (PZ) and rib-meristem. The cells in the CZ divide at a slower rate than cells in PZ. The CZ has been postulated to harbor stem cells, while the cells in the PZ are incorporated into making organ primordia. The rib-meristem cells are incorporated into developing stem. The SAM is a dynamic structure with cells constantly changing their position, without compromising the relative sizes of each of the domains. Though the anatomical and cytological studies have revealed the structure of the meristem, the dynamic nature is less understood. We are devising methods to visualize living cells in the developing meristem using confocal microscopy.

The cells of the SAM are deeply seated and are covered by developing organs at the periphery and so the ability to visualize such cells depends greatly on generating fluorescent markers and optimizing conditions for microscopy. One approach we have been using to completely expose the meristem for microscopy utilizes the diphtheria toxin to arrest the growth of young floral buds on the meristem flanks. Initial tests using plants expressing endoplasmic reticulum localized GFP under the constitutive 35S promoter have shown that we can clearly resolve individual cells to a depth of approximately 50 μm. In addition we also found that the diphtheria toxin system may be unnecessary in some cases where expression is particularly strong or localized in the central zone. For

example, the expression of GFP under the control of the CLV3 promoter that drives expression in the CZ was successfully monitored for several days in wild-type plants. In order to optimize cellular resolution, we have used various localization signals to target GFP to different sub-cellular, including the endoplasmic reticulum and nucleus. Our studies so far indicate that plants continue to develop under our imaging conditions, as revealed by morphological changes in shoot apex and the periodic onset of CLV3 expression in developing floral buds.

106. Cell division and displacement dynamics in developing meristems

G. Venugopala Reddy

A tight coordination between stem-cell division and displacement of their progeny has been proposed to be a major factor in regulating the size of stem-cell domain and in generating a radial pattern of the shoot apex. Thus far the studies related to analyzing cell behavior in the meristem are based on cytological staining methods on tissue sections. The studies so far have shown that the cells in the central zone (CZ) divide at a slower rate than cells in the peripheral zone (PZ). But these methods do not reveal the cell division and displacement dynamics. Therefore, generating a precise map of cell division and displacement patterns is central to understanding both cell-type specification and pattern formation in developing meristems. The aim of this project is to utilize live imaging techniques to assess and analyze the cell division and displacement patterns. The project involves labeling individual stem cells in the shoot apical meristem (SAM) and monitoring the propagation of an individual clone as it proceeds towards the periphery or into the rib-zone (RZ). The rate of propagation of the clone in different regions of the SAM should reveal the cell division rates. I am currently developing both the genetic methods of labeling cells and physical loading of lipophilic dyes such as DiI and its derivatives.

107. Using reverse genetics to analyze five genes in the CLE family

Catherine C. Baker

CLAVATA3 is a small-secreted protein that acts to reduce cell proliferation in the shoot meristem by activating CLAVATA1, a leucine-rich repeat receptor-like kinase. Twenty-four putative genes similar to *CLV3* have been identified in *Arabidopsis*; each member of this family is small (less than 400 bp) and is predicted to encode a protein with an N-terminal signal sequence. In additional, the predicted proteins share homology within a small C-terminal region, containing an invariant histidine, an invariant arginine, and a conserved glycine. This group of genes has been termed the *CLV3/ERS*-like (*CLE*) family, because of additional resemblance to embryo-surrounding region proteins in maize (1). We are interested in determining whether some of these predicted secreted proteins might act as ligands for one or more of the numerous receptor kinases in *Arabidopsis*. In collaboration with Jennifer Fletcher's lab at the Plant Gene Expression Center, we are investigating the function of five of these genes (*CLE11*, *12*, *16*, *17*, *18*) by using RNA interference, overexpression, and RNA *in situ* hybridizations. In addition, any fertile overexpression line with a visible phenotype would be mutagenized in order to identify downstream targets (such as receptors).

Reference

(1) Cock, J.M. and McCormick, S. (2001) *Plant Physiol.* **126**:939-942.

108. The role of auxin in positioning plant organ primordia

Marcus Heisler

In higher plants, organ primordia arise in a continuous predictable arrangement, or phyllotaxis, on the shoot apical meristem. The results of several studies have suggested that in plants, the hormone auxin may promote primordial growth and that its distribution may directly determine the site of primordium initiation. The aim of this project is to test this hypothesis directly and upon confirmation, to determine the molecular mechanism by which auxin is distributed in such a way.

In order to determine the distribution of auxin I, am trying to construct an auxin-responsive reporter gene that works in the meristem. To identify an appropriate promoter, I have used our microarray facility to identify genes that are induced in the meristem upon treatment with auxin. So far I have identified a moderate number of genes, including members of the previously characterized auxin induced IAA/AUX and GH3-like gene families, suggesting that this approach is working. Significantly, the expression patterns of most genes examined show high expression in regions predicted to form primordia. This result is consistent with the hypothesis that auxin is distributed within the meristem in a phyllotactic pattern. To test whether there is a causal link between auxin and primordia positioning, I plan to disrupt the auxin distribution in the meristem and test whether primordium initiation continues to follow the pattern of auxin distribution. This will be examined using live imaging techniques including confocal/multiphoton microscopy with multiple fluorescent proteins. The auxin distribution will be disrupted by using chemical inhibitors of auxin transport as well as by inducing sectors of cells that lack PIN1 auxin efflux activity.

Lastly, in order to understand how the pattern of auxin accumulation within the meristem is formed I am, in conjunction with Jeff Long, constructing auxin efflux carrier:GFP fusion proteins to allow the direction of auxin efflux from meristem cells to be determined. I am also assessing where auxin is synthesized by looking at the expression patterns of genes encoding auxin synthesis enzymes. Initial examination of PIN1:GFP localization within the meristem has revealed a highly interesting and complex pattern that together with knowledge of where auxin synthesis occurs and where auxin concentrations are high, may reveal a molecular mechanism responsible for plant phyllotaxis.

109. Identification of genes required for graviresponse in the *Arabidopsis* root

Elizabeth S. Haswell

In *Arabidopsis*, cell identity is determined by position. The force of gravity provides a basic polarity that informs cell identity and thereby influences the development, growth, and environmental response of the plant. When a wild-type root experiences a change in the gravity vector--if the plant is set on its side, for example--it responds by bending down. The root bends by differential distribution of the plant growth hormone auxin, resulting in increasing growth on the top side of the root, and decreased growth on the bottom side (1). Plant roots primarily perceive gravity through the sedimentation of starch granules in the columella cells of the root cap. *phosphoglucomutase* (*pgm*) mutants, which are unable to synthesize starch, have no starch granules and are insensitive to gravity (1). The molecular events that connect the sedimentation of amyloplasts and the redistribution of auxin are almost completely unknown.

We have undertaken a genetic screen to identify components of the gravity perception and signaling system in *Arabidopsis* roots. A number of loss-of-function screens for these same genes have been performed without much success, suggesting that the genes required for graviresponse are required for viability; we have therefore developed a gain of function screen. As the root is positively gravitropic but negatively phototropic, when seedlings are grown with light from the bottom the light and gravity vectors create opposing forces for the root. In a wild-type root, the graviresponse is stronger than the photoresponse, and a wild-type seedling will grow with both its shoot and its root facing down. Under the same conditions, a *pgm* mutant seedling grows with its shoot down and its root up, as the gravity response is absent and cannot counteract the negative phototropic response (1). We are screening an activation tag library for mutations that suppress the gravity response defect in the *pgm* mutant, allowing the root to grow down even in the presence of light from below.

References

(1) Friml *et al.* (2002) *Nature* **415**:806-809.
(2) Vitha *et al.* (2000) *Plant Phys.* **122**:453-461.

110. Intercellular trafficking of a meristematic transcription factor

Elizabeth S. Haswell

All of the stem, leaves, and flowers of a plant are derived from a population of stem cells called the shoot apical meristem (SAM). In the SAM, cell fate is influenced by a network of positional information provided by neighboring cells. Recent data suggests that in Arabidopsis, positional information may be communicated through the transport of transcription factors from the nucleus of one cell to the nucleus of another (1-4). Neither the nature of the route for movement nor the role of intercellular trafficking of nuclear factors in plant development is known. We hope to address these issues by screening for mutants unable to move transcription factors from cell to cell. Once we have identified genes whose function is required for intercellular trafficking, we will use the identity of these genes as an entry point for cell biological and biochemical analysis of the transport pathway. Furthermore, once we can prevent movement with mutations, we will be able to assess the importance and function of the process.

We are using the movement of the meristematic transcription factor SHOOTMERISTEMLESS (STM) as a model system for our screen. *stm* plants arrest growth at the seedling stage, lack a SAM and have fused cotyledon petioles. STM encodes a homeodomain transcription factor and is expressed in two regions of the SAM: the Central Zone (CZ) in the middle of the SAM, and the Peripheral Zone (PZ) in the region between the cotyledon primordia (4-6). It is thought that STM functions in the CZ to maintain the SAM, and in the PZ cause separation of the cotyledon petioles.

Interestingly, a 3.5 Kb fragment of the STM promoter drives expression only in the PZ, yet can complement the *stm* mutant phenotype (3,7). This result implies that the STM protein must move from the PZ to the CZ in order to maintain the meristem. Experiments to confirm that *STM* is only transcribed in the PZ, and but that the STM protein is found in both the PZ and the CZ, are currently underway. Seeds from this line will be mutagenized and M2 families will be screened for mutants that separate cotyledon petioles but are unable to maintain a SAM, indicating that STM is functional in the PZ but cannot move to the CZ.

References

(1) Sessions, A. *et al*. (2000) *Science* **289**:779-782.
(2) Nakajima, K. *et al*. (2001) *Nature* **413:**307-311.
(3) Fernandez, A. *et al.* (2001) in *11th International Conference on Arabidopsis Research,* Madison, WI.
(4) Barton, M.K. and Poethig, R.S. (1993) *Development* **119**:823-831.
(5) Long, J.A. *et al*. (1996) *Nature* **379**:66-69.
(6) Long, J.A. and Barton, M.K. (1998) *Development* **125**:3027-3035.
(7) Haswell, E. and Meyerowitz, E.M. - unpublished data.

111. Identification of endogenous plant proteins capable of intercellular movement

Elizabeth S. Haswell

Recent experimental evidence suggests that several *Arabidopsis* proteins are capable of moving from cell to cell in the plant (1-3). That these proteins are all nuclear factors involved in development implies that the intercellular trafficking of transcription factors provide a novel mechanism for intercellular communication in plants, perhaps imparting positional information or developmental cues. Before we can understand the function and potential regulation of this process, it must be characterized on a more basic level. At present, we do not know what proportion of plant proteins possess the ability to move from cell to cell, what processes these proteins

may act in, nor have we identified the cellular and molecular nature of the movement pathway or pathways. We are employing a genetic approach to identify a pool of endogenous plant proteins capable of robust cell-to-cell movement. By identifying a pool of nuclear proteins capable of cell-to-cell trafficking, we hope to: 1) determine if intercellular movement is a common or a specialized function of plant nuclear proteins; 2) identify a domain or signal sequence capable of conferring intercellular movement; 3) gain insight into the number and variety of developmental pathways that may involve intercellular trafficking; and 4) provide tools for the subsequent identification of the cellular pathway or pathways for movement.

In the construction of this screen, we are exploiting a two-component system involving two recombinant plant lines, the driver lines and the reporter lines (4). In the driver lines, a tissue-specific promoter promotes the expression of a synthetic transcriptional activator, LhG4; in the reporter lines, six copies of the LhG4 binding site are placed upstream of a cell-autonomous reporter gene. To identify plant proteins that move from cell to cell, we will screen a cDNA-LhG4 fusion library expressed from the GLABROUS 2 (GL2) promoter. The GL2 promoter is active in trichomes, unicellular hairs that grow on the leaves, stem, and sepals of *Arabidopsis*. This line will also carry a GFP-ER reporter transgene. If a cDNA fragment does not affect the function of LhG4, this line should cause trichome-specific GFP signal. However, if a cDNA fragment confers cell-to-cell movement on the LhG4 protein, GFP expression will be seen in the trichomes and in the surrounding cells of the leaf. We are currently testing the concept of the proposed screen by comparing GFP signal in lines carrying a driver line expressing LhG4 (GL2p::LhG4) to those carrying LhG4 fused to a viral movement protein (GL2p::LhG4-viral movement protein). We expect to see GFP expression both in the trichomes and in the surrounding leaf cells when LhG4 is fused to the viral movement protein.

References

(1) Sessions, A. *et al.* (2000) *Science* **289**:779-782.
(2) Nakajima, K., *et al.* (2001) *Nature* **413**:307-311.
(3) Fernandez, A. *et al.* (2001) in *11th International Conference on Arabidopsis Research,* Madison, WI.
(4) Moore, I. *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**:376-381.

112. Isolation of microRNAs from *Arabidopsis*

Eva C. Ziegelhoffer

Recent reports in the literature suggest an abundance of non-coding RNAs (ncRNAs) are encoded in animal genomes, providing a large class of genetic elements that do not code for proteins, and that cannot be annotated by searching for open reading frames. One of the most recently identified classes of ncRNAs from animals has been named microRNAs (miRNAs) due to its diminutive size of approximately 21 nucleotides. miRNAs are an exciting new collection of molecules with great regulatory potential. They are hypothesized to act in one of two general mechanisms. In the first case, they can act like known regulatory RNAs from *Caenorhabditis elegans*, *let-7* and *lin-4*, which are 21-22 nucleotide small temporal RNAs (stRNAs) and act as translational repressors by binding with imperfect complementarity to 3'UTRs of target genes involved in the progression of larval development. Alternatively, they could act like short interfering RNAs (siRNAs) which bind to and degrade endogenous and transgene mRNA upon RNA interference (RNAi)/post-transcriptional gene silencing (PTGS). The abundance of miRNAs isolated from different animal species inspired us to investigate the possibility that miRNAs are also present in plants. To identify miRNAs in plants, total RNA was isolated from various *Arabidopsis thaliana* tissues and then size selected using denaturing polyacrylamide electrophoresis. After ligation with linkers, the putative miRNAs were amplified by PCR, cloned and sequenced. Several putative miRNAs were isolated and are currently being characterized by two methods. In the first, putative precursors to the miRNAs are examined for possible secondary structures resembling double-stranded RNA, likely to be important for processing of miRNAs to their mature size. Secondly, expression of miRNAs and possible precursor RNAs are detected by Northern blots. The possibility of differential expression of miRNAs through development will be investigated by the testing of RNA from different tissue types.

113. Elucidation of GA signaling pathway in floral development

Hao Yu

Gibberellin (GA) plays an important role in many aspects of plant growth and development, such as seed germination, leaf expansion, stem elongation and floral development. It has been recently proposed that that GA regulates flowering time and floral organ identity by the involvement of five DELLA domain-containing GRAS transcriptional regulatory proteins (GAI, RGA, RGL1, RGL2, and RGL3) in the signaling pathway (1).

Current studies have revealed that *rga-t2 gai-t6* can almost totally rescued the late-flowering phenotypes of *ga1-3*, while the single *rga-t2* and *gai-t6* in ga1-3 background still shows significantly late flowering, indicating that GA promotes flowering by derepression of both *RGA* and *GAI* in the floral transition. Simultaneously, recent studies in flowering time control have shown that three possible genes, *SOC1*, *AGL24*, and *LFY* may be involved in the signal reception from GA promotion pathway. We are using the RGA- and GAI-GR systems in the *rga-t6 gai-t6 ga1-3* background to identify the potential point (s) where the GA signaling pathway is integrated in the multifactorial control of flowering time.

Although *rga-t2 gai-t6* can rescue the defect of flowering time in *ga1-3*, it still produces the same abnormal fertile flowers as *ga1-3*, which suggests that the GA signaling in floral organ formation is at least partially

separated from that in the control of flowing time. Thus, the other three signaling genes, *RGL1*, *RGL2,* and *RGL3,* possibly control the regulatory cascade in flower development. Recent studies have shown that *RGL1* and *RGL2* are strongly expressed in the floral organs. However, their single and double loss-of-function mutants have not shown clear visible defects in the development of floral organs. We will subsequently investigate the phenotype of the *rgl1 rgl2 rgl3* triple mutant and study the possible relationship between *RGLs* and other floral organ identity genes by expression pattern studies and genetic analyses. It is noteworthy that GA promotes *LFY* activity during flowering process, and *LFY* subsequently activates certain floral organ genes. We are curious about the mechanism: whether *RGLs* regulate LFY, and thus control the activity of floral organ genes, or whether they directly control floral organ genes in parallel with LFY activity.

Reference

(1) Dill, A. and Sun, T. (2001) *Genetics* **159**:777-785.

Publications

Kishimoto, N., Sakai, H., Jackson, J., Jacobsen, S.E., Meyerowitz, E.M., Dennis, E.S. and Finnegan, E.J. (2001) Site specificity of the *Arabidopsis* METI DNA methyltransferase demonstrated through hypermethylation of the superman locus. *Plant Mol. Biol.* **46**:171-183.

Long, J., Woody, S., Poethig, S., Meyerowitz, E.M. and Barton, M.K. (2002) Transformation of shoots into roots in *Arabidopsis* embryos mutant at the *TOPLESS* locus. *Development* **129**:2797-2806.

Meyerowitz, E.M. (2001) Prehistory and history of *Arabidopsis* research. *Plant Physiol*. **125**:15-19.

Meyerowitz, E.M. (2002) Plants compared to animals: The broadest comparative study of development. *Science* **295**:1482-1485.

Shapiro, B.E., Mjolsness, E.D., Levchenko, A., Wold, B. and Meyerowitz, E.M. (2002) CELLERATOR: Automated model generation using a palette enterable arrow based notation. *Bioinformatics.* Submitted for publication.

Wagner, D. and Meyerowitz, E.M. (2002) SPLAYED, a novel SWI/SNF ATPase homolog controls reproductive development in *Arabidopsis. Curr. Biol*. **12**:85-94.

Wolpert, L., Beddington, R., Jessell, T., Lawrence, P., Meyerowitz, E.M. and Smith, J. (2002) *Principles of Development,* Second Edition, Oxford University Press, 542 pp.

Professor of Biology: Ellen V. Rothenberg
Member of the Professional Staff: Rochelle Diamond
Senior Research Fellow: Michele Anderson
Postdoctoral Scholars: Satoko Adachi, Jing Chen, Elizabeth David, Angela Weiss, Mary Yui
Graduate Student: Christopher Dionne, Luigi Warren, Mark Zarnegar
Research and Laboratory Staff: Alexandra Arias, Robin Condie, Rochelle Diamond, Parvin Hartsteen, Rashmi Pant

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Summary: The research problems addressed in the Rothenberg laboratory concern the mechanisms that drive hematopoietic precursors to develop into T lymphocytes. At least two major phases of this process can be distinguished: (1) early, T-cell receptor-independent events, involving the developmental choices of whether to commit to the T lineage at all; and (2) later, T-cell receptor-dependent events, which determine whether or not particular immature T-lineage cells express antigen receptors that will be useful to the organism. Most current work in the Rothenberg group focuses on the early phase. The cells that undergo the events leading to T-lineage commitment are pluripotent hematopoietic precursors that can initially generate natural killer lymphocytes and dendritic cells as well as T cells, and possibly B cells as well. These alternative developmental potentials are lost as T-cell genes are increasingly turned on and non-T genes, which are initially expressed in the precursors, become silenced. A major question we are addressing is how specific gene expression changes result in T-lineage commitment. We have been addressing this both in terms of the developmental impacts of several critical transcription factors and in terms of finding specific *cis*-regulatory sequences of target genes through which these regulators work.

The past year has been notable for several new research initiatives in the lab. We have recently made extensive use of retroviral transduction and *in vitro* lymphoid differentiation systems to test the effect of perturbing the normally tight developmental control of essential T-lineage transcription factors. The results have shown that successful T-lineage commitment depends on a delicate balance of transcription factor levels, and that overexpression of any of the essential factors can abort this process. Moreover, different essential transcription factors show distinctive temporal windows for perturbation of T-cell development, and distinctive effects on the ability of uncommitted cells to take specific alternatives to the T-cell path. Now, in the past year, we have begun systematically dissecting these developmental effects in terms of effects on transcription of multiple target genes. The results have enabled us to construct a provisional draft network of regulatory interactions in the T-lineage commitment process. This is under continuous empirical revision, but already acts as a source of specific predictions for focused reverse-genetic experiments. The draft network thus provides an armature for understanding a highly complex vertebrate innovation, the T-cell developmental program.

The network shows that among the targets most strongly affected by misexpression of key transcription factors are genes encoding other transcription factors or stage-specific growth factor receptors, i.e., genes which themselves have further regulatory impact. Such genes, for example the gene encoding PU.1, are particularly attractive to analyze at a molecular level to determine the specific *cis*-regulatory sequences and regulatory inputs that control their *in vivo* expression patterns. We have therefore put strong new emphasis on mapping and analysis of direct *in vivo* protein/DNA contacts by chromatin immune precipitation and *in vitro* binding assays.

The network analysis promises to be strengthened by advances in two other areas. First, our gene discovery project to identify the cohort of transcription factors that are active during T-lineage commitment has moved into a new phase, with a comprehensive new set of sequence data on >1300 selected cDNAs and extensive progress on determining the patterns of expression of the most interesting ones, in quantitative detail. Second, we are testing a possible cell line model for T-lineage commitment: if this proves to be mimicking normal T-lineage commitment, it will be a powerful tool for defining the molecular alterations that commitment represents. These advances will need to be joined by development of additional technology in order to confirm the roles of particular genes in normal T-cell development. Ultimately, the network defined by high-level perturbation will need to be confirmed by loss-of-function analyses of the same genes. Thus we are attempting to adapt morpholino-derivatized antisense oligonucleotide or shRNA (RNA interference) approaches to ablate expression of regulatory genes at specific stages in T-cell precursor differentiation.

These new initiatives in the lab enrich the context for our continuing projects.

(1) We are investigating the possibility that the precursors of different T-cell subsets, including NKT cells and TCR-γδ T cells, may diverge early in T-cell development, distinguishing possible subset founders by cell surface markers and expression of an IL-2 regulatory sequence transgene, and testing their developmental potential in thymic organ cultures.

(2) IL-2 is a gene that is inducible in all subsets of naïve T cells but in few if any other cell types. It provides access to mechanisms that make genetic loci accessible in chromatin of T-lineage cells, as a heritable property that may be distinct from active transcription. The distal *cis*-regulatory IL-2 sequences, which we have previously found to exhibit some locus-control-like activity, are now shown to correspond to an extended region of constitutive histone acetylation in IL-2-expressing cells, which is not acetylated in nonexpressing cells. We are now identifying transcription factors that bind to this region *in vivo*.

(3) The genes used in early development can be linked with regulation of later T-cell function. The multigenic disease, autoimmune diabetes, presents as a

failure of mature T-cell tolerance, but turns out to include a failure of the developmental checkpoint in the thymus that marks the end of the period of lineage commitment, known as β-selection. We are mapping the relevant loci in backcrosses between control and diabetes-prone mouse strains and monitoring genes involved in β-selection to analyze the molecular basis of this checkpoint violation.

(4) To extend our studies of immune system evolution, we have defined the cartilaginous fish members of the EBF, GATA-2/3, and Runx transcription factor families and have begun to determine the expression pattern of some lamprey hematopoietic transcription factors. These studies, with our previous results on the Spi/PU.1 subfamily of Ets transcription factors, give a richer and more complex perspective of the way the transcription factor-coding genes used in immune-cell development underwent duplication and diverged in function between basal vertebrates and mammals.

114. Constitutive expression of PU.1 in fetal hematopoietic progenitors blocks T-cell development at the pro-T stage: Mutational analysis of PU.1 domains

Angela Weiss, Michele K. Anderson

PU.1 is a powerful transcription factor that is known to be essential for the development of macrophages, granulocytes, B cells, and fetal T cells. It is expressed at the earliest stages of thymocyte precursor development, but is sharply downregulated just during the stages when T cell lineage commitment occurs. As described last year, this downregulation is functionally important for T cell development to occur, as we found by using bicistronic retroviral vectors expressing PU.1/GFP or GFP only to infect fetal liver-derived hematopoietic precursors. PU.1/GFP$^+$ or GFP$^+$ cells were sorted and allowed to differentiate in fetal thymic organ cultures (FTOC), which allows for T-cell development, or high oxygen submersion culture (HOS) supplemented with cytokines, which also allows for development of macrophages, B cells and NK cells. We could show that PU.1 overexpression leads to an overall diminution in lymphoid precursor numbers in the FTOC system and an inhibition of development in very early stages of T-cell development. Transduced T-cell precursors that express PU.1 at a modest level can progress through the early stages of T-cell development, but not beyond the DN3 pro-T cell stage, just before β-selection. This is at the same stage when normally developing T cells shut off PU.1 expression and undergo T-cell lineage commitment. If cells are forced to express PU.1 further in a microenvironment that promotes T-cell development, the cells are eliminated. We also found that downregulation of PU.1 is also essential for B-cell development in HOS cultures. In contrast, under the same culture conditions we not only see good cell survival, but also differentiation of PU.1 overexpressing precursor cells taking the myeloid/ macrophage pathway.

PU.1 consists of many different domains that mediate its interactions with diverse transcriptional partners. Different interactions are implicated in different developmental roles. In order to investigate which of these domains is essential for the effects on developing T cells, we created a number of different mutants that we are testing in our system. In one of these mutants the DNA binding ability is abolished by introducing a single amino acid change. When this mutated form of PU.1 is introduced into our FTOC system, overexpressing cells are able not only to survive in numbers equal to control cells, but also to differentiate into T cells as well as untransduced cells. This indicates that the integrity of the DNA binding domain is important for PU.1 to exert its effects on T-cell development.

In a second mutated form of PU.1, PU.1Δ75, amino acids 75 to 100 were deleted. This removes an important transactivation domain that has been shown to be vital for macrophage development and to inhibit B-cell development. Others have reported that PU.1Δ75 overexpression allows B-cell development in optimal conditions, where overexpression of the wild-type form does not. Cells that overexpress this mutated form of PU.1 and develop in our HOS system still cannot develop into B cells, however. Instead we see a higher percentage of Mac-1-expressing cells. However, PU.1Δ75 overexpression has a more selective effect on developing T cells in the FTOC system. Interestingly, PU.1Δ75 overexpressing cells do proliferate as well as control cells, yet still show the same developmental block as PU.1 overexpressing cells. This indicates that the two effects of PU.1 overexpression that we see during T-cell development are controlled by two different domains.

Further experiments with additional mutated forms of PU.1 will give a more complete insight into functional activities of different domains of PU.1 during T cell development.

115. Mapping network elements for early T-cell development

Michele Anderson, Gabriela Hernandez-Hoyos, Christopher Dionne, Alexandra Arias, Ellen Rothenberg

PU.1 and GATA-3 are both essential transcription factors expressed at the time that hematopoietic precursors undergo commitment to the T-cell lineage or to alternative lineages in the thymus. In order to understand the roles of these factors in T-cell development, we manipulated their levels of expression in fetal precursor cells by using retroviral constructs that encoded either PU.1 or GATA-3 in addition to GFP as a marker. Cells infected with vector expressing GFP only were used as controls so that infected cells could be isolated by fluorescence-activated cell sorting. As described in the previous abstract, the developmental impacts could be measured by placing the GFP$^+$ cells in fetal thymic organ culture for differentiation into T cells. To explore the mechanisms responsible for the developmental effects, the GFP$^+$ cells were maintained in short-term *ex vivo* culture for analysis of changes in gene expression. The SCID.adh cell line, which closely resembles the early DN3 stage of thymocyte development, was also infected with PU.1 retroviral constructs and used to study gene expression.

PU.1 is normally expressed early in T-cell development, but is shut off at the time that commitment to the T lineage occurs. As described in the previous abstract, when we forced PU.1 to stay on in precursors past the point at which it is normally repressed, we observed

first a great decrease in the percentage of GFP^+ cells as compared with controls. These PU.1-expressing cells did develop to the DN3 stage (one stage past commitment), but caused a complete block at the DN3 to DN4 transition, which is dependent on successful rearrangement and expression of the TCR β chain (β selection). Furthermore, neither αβ T cells nor γδ T cells could be generated from the PU.1-expressing cells. GATA-3, unlike PU.1, is expressed throughout T-cell development, and is essential for the production of T cells from the earliest stage. However, GATA-3 overexpression also caused a sharp decrease in cell number and surprisingly blocked development at the DN1 stage, even earlier than the PU.1 block. In rare cases, a few GATA-3 expressing cells broke through the block and transitioned from DN3 to DN4, but again no T cells of either the αβ or the γδ lineage developed. These results emphasize the importance of regulating both the timing and the levels of critical differentiation factors in T-cell development, and establish the repression of PU.1 as a requirement for the development of mature T cells.

Infections of SCID.adh cells, fetal liver-derived stem cells, or fetal thymocytes were also performed to generate GFP^+ cells that were then sorted and used as template in quantitative realtime PCR assays to evaluate potential targets of PU.1 or GATA-3. These studies revealed that PU.1 can downregulate several genes that are essential to the DN3 to DN4 transition, while upregulating genes associated with the macrophage lineage. High levels of GATA-3 also downregulate several genes involved in TCR recombination, and upregulate genes associated with a stem cell or erythro-megakaryocytic fate, suggesting that a "misinterpretation" of GATA-3 as either GATA-1 or GATA-2 is possible in this context when expression levels are elevated. Importantly, GATA-3 can downregulate PU.1 in this context as well, and this interaction may provide one mechanism of lineage commitment in normal T-cell development. The architecture of these interactions has been diagrammed as a transcriptional network for T cell specification and commitment. This network provides a mechanistic view of how differential activation of specific nodes can lead to coordinated outcomes such as fate specification and lineage-specific differentiation programs.

116. Differential regulation of two HEB isoforms from distinct promoters in early T-cell development

Michele K. Anderson, Ellen V. Rothenberg

The class A bHLH transcription factors (E2A, HEB, and E2-2) play essential roles in many developmental processes, including lymphocyte development. A novel isoform of HEB was identified during a differential screen for transcription factors expressed during early T-cell development. This isoform, HEBAlt, is transcribed from an internal promoter between exons 8 and 9 of the full-length HEB gene, and contains a short novel amino acid sequence at the N-terminus. HEBAlt lacks one of the transactivation domains contained within the canonical form of HEB (HEBCan), and also lacks a putative nuclear localization signal sequence. The genomic structure of this gene is conserved between human and mouse, and is also similar to that found for the human and mouse E2-2 genes. Quantitative RT-PCR analysis of sorted postnatal T-cell precursors was conducted to assess the distribution of the bHLH isoforms, the Id family negative regulators, and other potential interaction partners at different developmental stages. These results indicate that HEBCan, E2A, and E2-2 are all expressed throughout the course of T-cell development at varying levels. In contrast, HEBAlt is upregulated in the earliest thymic precursors, is expressed in DN2 and DN3 thymocytes, and is then shut off permanently at β selection. bHLH interaction partners SCL and Id-2, which usually act as negative regulators, are expressed at high levels in DN1, are sharply downregulated at DN2, and are completely off by DN3, whereas the negative regulator Id-3 is transiently induced after β selection. Id-2 is expressed at very high levels primarily in the thymic pre-NK (natural killer) cells, which agrees with the observation that NK cell development requires Id-2. Retroviral expression of HEBAlt or HEBCan in fetal liver-derived precursors placed into fetal thymic organ culture show that both isoforms inhibit proliferation but that neither one fully blocks production of either αβ or γδ T cells. HEBAlt, however, does cause a partial block at DN1 that resembles the phenotype of $E2A^{-/-}$ thymocytes, suggesting that HEBAlt may specifically interfere with E2A function. HEBAlt overexpression blocks B-cell development while sparing NK precursors, which also supports a role for HEBAlt in partially antagonizing bHLH function.

117. Thymocyte development in c-Fos- and FosB-deficient mice: Assays of cell autonomy

Angela Weiss, Fei Chen, Robin Condie, Alexandra Arias, Rochelle Diamond

The expression of the Fos family transcription factors, c-Fos and FosB, which are components of AP-1 heterodimers, is subject to sharp regulation at two checkpoints during thymocyte development. Both can be induced in $CD4^-CD8^-$ double-negative (DN) and $CD4^+$ or $CD8^+$ single-positive (SP) cells, but not in cortical $CD4^+/CD8^+$ cells. C-Fos and FosB might therefore play an important role in the activation events involved in thymocyte development and function.

To investigate this question, we studied thymocyte development in c-Fos- and FosB-deficient mice. The Fos family mutants examined before this have not shown a definitive T-cell developmental phenotype. *FosB*$^{-/-}$ mice are viable and anatomically normal. In contrast, *c-fos*$^{-/-}$ mice are deformed by osteoporosis that results in extramedullary hematopoiesis, stunted growth, and failure of tooth eruption. In spite of those differences both single-gene knockout genotypes can support essentially normal T-cell development. These phenotypes could be due to two reasons: (i) as members of the same transcription factor family, c-Fos and FosB could substitute for each other; or (ii) c-Fos and FosB do not play any significant role during T-cell development. To distinguish between these possibilities we have crossed the c-Fos and FosB mutants to obtain heterozygous and homozygous double knockout mice.

As reported last year, in these double knockout mice we could show a gene dosage-dependent, age-

dependent inhibition of T-cell development with a clear restriction point at the $CD44^+CD25^+$ (DN2) stage: cells with the $CD25^+CD44^-$ or $CD25^-CD44^-$ phenotypes (DN3 and DN4, respectively) are greatly reduced while the most primitive $CD25^-CD44^+$ cells (DN1 cells) constitute 70-85%. This skewing is not seen in any of the single homozygous knockout mice. The phenotype of the double-mutant mice raises the question how much the altered microenvironment does contribute to the outcome. There are two different types of microenvironment that are important for T-cell differentiation: the thymic stroma and the prethymic hematopoietic microenvironment, which is seriously and progressively affected by osteoporosis in the mutants.

To address this question we created bone marrow chimeras. Normal mice were irradiated and then injected with splenocytes or bone marrow cells of homozygous or heterozygous double-knockout mice. Interestingly, about 70% of the mice that were injected with bone marrow of homozygous double knockout mice showed a fully reconstituted T-cell development. Even splenocytes of the same donor could reconstitute, but at a lower frequency. This suggests that the block in T-cell development seen in the mutant animals is rather due to the altered microenvironment than to a block in the T-cell precursors themselves.

118. Analysis of the role of GATA-3 in prethymic hematopoietic precursors using a tamoxifen-dependent GATA-3 fusion gene

Alexandra Arias, Elizabeth-Sharon D. David

We are interested in studying the role of GATA-3 in T-cell lineage commitment. GATA-3 is a zinc-finger transcription factor that is required at various stages in T-cell development, and its expression is maintained at some level throughout. GATA-3 and its related family members, GATA-1 and GATA-2, have been implicated as essential for various blood-cell lineage decisions. We would like to understand the regulatory basis of this lineage choice in T-cell development. What mechanisms are involved to cause hematopoietic precursors to restrict developmental potential until lineage commitment is established? In particular, what is the importance of GATA-3 in this process?

Our lab has shown that constitutive over-expression of the transcription factor GATA-3 causes a block in hematopoietic stem-cell development, and a partial block in lymphoid progenitor development. Overexpression of GATA-3 in fetal liver populations enriched for hematopoietic stem cells causes a severe inhibition of proliferation or loss of viability within the transduced population. The harsh prethymic effect may obscure any specific effects of GATA-3 activity in the earliest intrathymic stages of T-cell development.

To compensate for this inhibition, we have utilized a system that allows for conditional activation of GATA-3 within this progenitor population. We have generated hormone-inducible chimeric protein by fusing the coding sequences of GATA-3 and the estrogen receptor. The fusion protein is maintained in the cytoplasm until the addition of an estrogen analog (tamoxifen). This causes the translocation of the chimera to the nucleus in a tamoxifen dosage-dependent manner. This not only has the advantage of regulating the effective levels of GATA-3, but gives temporal control of its nuclear activation as well.

Our system has been confirmed by analyzing the localization of the GATA-3 through confocal microscopy. Our system has been very useful in controlling GATA-3 expression within progenitor populations of interest. It furthermore has helped to initiate studies of the interaction of GATA-3 with other transcription factors within these hematopoietic progenitor populations and ultimately the implications of GATA-3's role as these progenitors transition into T cells.

119. Transcription factors involved in early thymocyte development: Gene discovery

Elizabeth-Sharon David, Michele Anderson, Lee Rowen, Gillian Giorgio†, Lee Hood**

The transcription factors that are currently known to be important for T-cell development leave considerable gaps in our understanding of the mechanism underlying the transition from pluripotent stages, when the precursors can give still rise to non-T progeny, to committed stages when the cells can generate T-lineage progeny only. To identify a broader set of transcription factors that could help to engender these commitment changes, we are differentially screening a robotically arrayed library of about 72,000 cDNAs from immature thymocytes. This library was derived by Michele Anderson from a mutant mouse strain (SCID) in which most thymocytes are arrested at the DN2 stage, immediately preceding commitment.

The master SCID thymus library was first screened with probes for conserved protein domains involved in DNA binding, chromatin modification, RNA binding, or protein-protein interactions. A 1380-clone rearray was prepared by selecting the positive clones from the master arrayed library. These clones, collected into 384-well plates and spotted onto high-density filters by the Q-Bot facility, were also completely sequenced by high throughput methods at the Institute for Systems Biology. As expected, not all the clones encode transcription factors: many of the rearray clones clearly encode signaling mediators, adaptors, chromatin proteins, and RNA-binding proteins, while another large fraction are unknown except as extended matches to ESTs. However, clones encoding 84 interesting transcription factors were isolated. Several of them have not hitherto been known to be involved in T-cell development. Among these genes, the zinc-finger family of transcription factors (27 different molecules) seems to be prominently implicated. Other transcription factors known to be involved in T-cell development from the HMG box, bHLH, Ets, GATA, and POU domain families were also recovered.

To identify an initial spectrum of genes preferentially expressed in early T-cell precursors, the rearray was screened with complex RNA probes from different stages of T-cell development and from hematopoietic stem-cell-like cell lines. We have now begun a more accurate analysis of gene expression using quantitative real-time RT-PCR (qPCR) to compare cDNA samples from sorted populations of cells from different DN stages, more mature T cells, and a large panel of non-T

tissues and hematopoietic cell fractions. The approach is validated by our finding that the gene that is most prevalent in our rearray as well as in differential screens is the known T-cell transcription factor Tcf1. Tcf1 is known to have several different possible splice isoforms. We have conducted Q-PCR using primers across alternately utilized exons and find that nearly all isoforms of Tcf1 are expressed most strongly in the T-lineage cells, with highest expression in a subset of the DN1 cells. Of the zinc-finger genes we now have evidence that *mzf-22, zfp95, KAB-1, mKr1* and *mKr5* are developmentally regulated during T-cell development. Of these, zfp95 is upregulated in the DN3 stage, just after commitment. Among other genes tested like *Rbak, Chromobox4* and *Period*, we found that *Period* is expressed at its highest in DN3, but it is at its lowest in DN2.

We plan to use the rearray as a "reagent" in the future, both to screen different stages of T-cell development for their expression of transcription factors and to determine expression patterns of novel candidate regulatory genes that were not previously known to be involved in T-cell development.

**Institute for Systems Biology, Seattle, WA*
†Undergraduate, SDSU, San Diego, CA

120. Post-transcriptional gene silencing strategies to test loss-of-function of transcription factor genes

Luigi Warren

The draft network of regulatory interactions that has been assembled in the Rothenberg laboratory so far relies entirely on gain-of-function perturbations. While identifying genes that can become targets, direct or indirect, of PU.1 or GATA-3 at high expression levels, the retroviral transduction approach does not necessarily identify physiological targets of these factors at their normal low levels of expression. Thus a complementary loss-of-function strategy is critical for the assessment of true physiological regulatory linkages.

I am currently investigating approaches to Post-Transcriptional Gene Silencing (PTGS) in thymocytes and T-cell lines. My work is focused on two strategies: transient transfection using antisense morpholinos; and chromosomal integration of retroviral constructs expressing short hairpin RNAs (shRNAs). Morpholinos present a technically easy, rapid-turnaround method of carrying out gene knockdown experiments. However, cells of the T lineage are notoriously hard to transfect, and it has yet to be shown that they can be persuaded to take up morpholinos in sufficient titers to make this a viable antisense strategy. My experiments to date suggest that the transfection efficiency into even robust, fast-growing lymphoma lines is at least an order of magnitude less than in proven morpholino targets such as fibroblasts. Nevertheless, since the mRNAs for the transcription factors we are interested in are typically present in low copy number, it is still possible that we may be able to use morpholinos to achieve useful knockdown. The transgene approach probably offers more hope for a long-term solution to the problem of inducible gene silencing. Until recently, RNA interference had proved ineffective in mammalian cultures, owing to the cells' generalized, interferon-based response to challenge with dsRNA. In the past few months a number of workers have reported successful PTGS using siRNAs (short interfering RNAs) that resemble intermediates produced in the RNAi pathway. These double-stranded, 22-nt oligos seem to fly "under the radar" of the interferon-mediated defenses. Self-hybridizing stem-loop transcripts called "short hairpin RNAs" (shRNAs) have also been found to trigger RNA interference. The siRNAs or shRNAs can be delivered by transient transfection, but a better solution would be to knock in transgenes that express the RNAs continuously. My short-term objective is to show that we can use the retroviral vector MSCV to introduce an shRNA-expressing transgene into our cell lines. If PTGS can be demonstrated, I plan to turn my attention to transduction of primary cells, to the development of more sophisticated shRNA vectors supporting inducible knockdown, and to regulatory network perturbation experiments.

121. Identification of regulatory elements for PU.1 expression in hematopoiesis

Jing Chen

PU.1 is a member of the *ets* family of transcription factors that is expressed exclusively in hematopoietic cells. Knockout studies have shown that PU.1 is required for the normal development of many blood-cell lineages, yet overexpression of this transcription factor in erythroid cells lead to erythroleukemia. Studies in Dr. Rothenberg's group focus on T-lymphocyte development. PU.1 is expressed at the earliest stages of thymocyte precursor development (DN1, DN2), but is sharply downregulated just during T-lineage commitment. Forced expression of PU.1 in fetal hematopoietic progenitors blocks T-cell development at the pro-T cell stage. Thus, how the PU.1 expression is regulated is a critical question for us to understanding T-lineage commitment and other hematopoietic cell differentiation. To address this question, we first started to identify *cis*-regulatory elements in PU.1 genomic region by using the DNase hypersensitivity assay. We found eight potential sites of DNA/protein interactions in those hematopoietic cell lines expressing PU.1, and only three of those sites were shared by non-PU.1 expressing hematopoietic cell lines. Some of the sites confirm reports from other groups, but one cluster of sites specific to the PU.1-expressing cells has never been described before. All of the regions of hypersensitivity in the 5'-flanking region appear to match islands of sequence conservation in the murine and human genes. Using chromatin immunoprecipitation assays (ChIP), we identified PU.1 itself and a repressive regulator CtBP as proteins interacting *in vivo* with the PU.1 5' flanking region which corresponds to one of the DNase hypersensitive sites. Currently we are testing whether forced expression of PU.1 can lead to opening its endogenous locus in two non-PU.1 expressing hematopoietic cell lines. Furthermore, we are looking for the factors interacting with CtBP that may provide a mechanism to understand how PU.1 is silenced during T-lineage commitment.

122. Identifying PU.1 regulatory mechanisms in developing T cells

Mark Zarnegar

In order to understand how PU.1 expression is controlled, great effort is being made to identify upstream regulatory domains. Through the use of mouse/human sequence comparisons (FamilyRelations, developed by C. Titus Brown *et al*.), potential upstream regulatory domains of the PU.1 gene were identified. These correspond well with the regions showing specific DNase hypersensitivity as described in the previous abstract. In chromatin immunoprecipitation assays, these regions were also found to be associated with hyperacetylated histone 3 (H3). H3 hyperacetylation is a sign of open chromatin structure and thus potentially active regulatory function. Most of the upstream domains are associated with hyperacetylated H3 only in PU.1-expressing myeloid cells, not in early T cells; however, one of the upstream domains shows H3 hyperacetylation both in myeloid cells and in T cells at the DN3 stage. Myeloid cells normally express high levels of PU.1 whereas T cells at the DN3 stage must turn off PU.1 or fail to develop further. This suggests that this domain could be engaged in the cell-specific regulation of this gene in T cells vs. myeloid cells.

DNA sequence analysis across this region indicates several potential binding sites for transcription factors known to affect hematopoietic lineage decisions including: Runx1, Ets, C/EBP, c-rel, and c-myb. Of particular interest are the Runx1 and Ets binding sites. Runx1 (CBFα2, AML1, PEBP2αB) is highly expressed in thymocytes and exists as multiple isoforms with varying protein interactions and DNA binding affinity. Electrophoretic mobility shift assays (EMSA) have produced potentially T cell specific bands containing RUNX1 and Ets proteins. Identifying which runx1 isoforms and Ets family members are involved with the complexes binding this region may provide insight into how this region may be differentially regulated in T cells versus other hematopoietic lineages.

The regulatory functions of this and other upstream regions will be examined in transgenic animal studies. By creating a "knock in" mouse, with GFP replacing PU.1, we will be able to follow GFP expression patterns in a developing embryo as controlled by normal PU.1 regulatory elements. By creating animal strains with deletions for specific regulatory regions or carrying mutations in the binding sites of relevant transcription factors, we can monitor the changes in GFP expression versus the normal knock-in mouse.

123. Examining thymocyte development and commitment with multiple clones of the SCID.adh thymic cell line

Christopher J. Dionne

Spontaneously arising diversity in a cell line appears to provide a novel tool to address the mechanisms involved in commitment to the T lineage in the early stages of thymic development. PU.1 is necessary for T development but when expressed ectopically *in vivo* some cells respond by inhibiting T development and initiating a myeloid program, becoming macrophage-like. This response seems both dependent on the stage of commitment of the cells and on the levels of PU.1 present. The SCID.adh cells similarly respond in a clone-dependent and dose-dependent manner to PU.1. Forced expression of PU.1 leads to two distinct kinds of responses in individual cells of the SCID.adh cell line, a thymic lymphoma that appears to represent the stage of thymocyte development when T lineage commitment occurs. Most of the cells are unaffected by PU.1 expression, while others with only slightly higher levels of PU.1 undergo concerted shifts in gene expression toward a more myeloid-like pattern. To investigate the mechanism responsible, we have established subclones of SCID.adh cells. These subclones appear to represent unique cell lines with stably different levels of commitment, as measured by response to PU.1 overexpression. This means that there are heritable differences between clones that control access to the target genes that PU.1 can regulate or the ability of PU.1 to work at all.

Currently I am characterizing and comparing the SCID.adh subclones as a system for analysis of early T development; examining RNA levels of T and non-T genes and transcription factors via realtime quantitative PCR, examining surface protein expression and assaying the responses to various factors of interest. The cells express many of the same transcription factors as primary pre-T cells, while there is little variation yet observed between the subclones. The similarity between PU.1-sensitive and PU.1-insensitive clones rules out trivial explanations of their different responses. It also indicates that the cells will be good candidates for use in a subtractive hybridization assay to examine factors involved in "plasticity" vs. "commitment." Concurrently we are using the SCID.adh subclones to examine the effects of the transcription factor PU.1 to an extent that would be extremely difficult *in vivo*. Future work will center on examining the characteristics involved in individualizing the subclones, examining the effects of various transcription factors, and using the information to assay for corollaries *in vivo*.

124. The developmental potential of DN1 thymocyte subsets

Mary Yui, Rochelle Diamond

Molecular biological analyses of T-cell differentiation ultimately depend on an accurate picture of the precursor/product relationships among putative T-cell precursor subsets. While the general outlines of these relationships are now well established, the earliest intrathymic precursors remain incompletely defined. Before T-cell receptor rearrangement, T-cell precursors in the thymus differentiate through a series of stages defined by cell surface markers: from DN1 ($CD44^+CD25^-$) to DN2 ($CD44^+CD25^+$) to DN3 ($CD44^-CD25^+$), whereupon cells must rearrange a T-cell receptor or die. The DN1 cells have been widely treated as a homogeneous population representing the most immature precursors. However, starting with precursor-enriched thymocytes isolated from SCID, Rag-deficient or $TCR\beta^{-/-}\delta^{-/-}$ double-mutant mice, we have been able to show that DN1 cells consist of at least three phenotypically distinct populations. These populations are also distinguished by their gene expression patterns and, as shown in our recent experiments, by their

in vitro developmental potential when transferred into fetal thymic organ culture (FTOC). We have previously shown by RT-PCR analysis that the DN1 cells have a major division in gene expression patterns between the $Sca1^{+}NK1.1^{-}$ and $Sca1^{-}NK1.1^{+}$ population. Furthermore, the $Sca1^{+}NK1.1^{+}$ population can be further divided by Thy-1 expression. We have now shown that a very minor subset of the DN1 cells, those that are $Sca1^{+}NK1.1^{-}Thy1^{low}$, is most enriched for cells with a potential to develop into T-cell precursors. These cells upregulate CD25, HSA and Thy1 and downregulate CD44 in FTOC, mimicking the *in vivo* development of T-cell precursors. In contrast, neither the $Sca1^{+}Thy^{high}$ nor the $Sca1^{-}NK1.1^{+}$ populations were capable of producing $CD25^{+}HSA^{+}$ DN2 or DN3 cells. To more precisely characterize the true precursor population, we have further subdivided the Thy^{low} population by use of additional markers including CD4, B220, and IL7R. These subsets can now be purified for a definitive test of developmental potential in FTOC and correlation with diagnostic gene expression patterns.

125. Differential regulation of IL-2 expression in γδ and αβ T-cell subsets

Mary Yui

IL-2 is an immunoregulatory cytokine whose expression is tightly regulated and generally restricted to activated T cells. We previously reported the identification of an upstream regulatory region of the murine interleukin 2 (IL-2) locus with at least 13 DNaseI hypersensitive (DH) sites. Using 8 kb of upstream sequence (which included most of the DH sites) to drive expression of GFP conferred position-independent transgene expression, while the 2 kb proximal to the promoter alone did not. GFP expression was found to be highly inducible in mature αβ T cells. However, in an apparent deviation from the regulation of the endogenous IL-2 gene, a particularly high percentage of transgenic γδ and NKT cells were spontaneously GFP-positive in thymus and spleen.

To determine whether differences in GFP expression between T-cell subsets accurately reflects differences in IL-2 expression at the RNA level, γδ, αβ, and αβ-NKT cells were FACS sorted from the spleens of four different 8 kb transgenic lines. RNA was extracted from the sorted cells after stimulation *in vitro* for 16 h with antibodies to the CD3ε molecule of the T-cell receptor complex and to the costimulatory molecule, CD28. Relative amounts of GFP and IL-2 mRNA were determined using real-time quantitative RT-PCR. While stimulated αβ T cells and αβ NKT cells make abundant IL-2 mRNA, γδ cells make relatively less. However, γδ cells from all lines make relatively more GFP mRNA than do αβ cells. Overall, the ratio of GFP to IL-2 was significantly higher in γδ than in NKT or conventional αβ T cells. Thus, the transgene reveals that αβ and γδ T cells regulate IL-2 differentially at the mRNA level. We are investigating whether this is at the level of transcriptional activation or of mRNA stability.

Additionally, our results provide hints that IL-2 expression in αβ and γδ T cells may respond to different *cis*-regulatory elements. To determine the role of the prominent DNaseI hypersensitive (DH) site found at approximately –4.5 kb, we deleted 880 bp of DNA sequence flanking this DH site and made new transgenic mice with this construct (IL2pΔ4). Only 2/5 lines obtained with this new construct expressed the transgene well upon stimulation (compared with 10/11 for the 8 kb construct), indicating that key locus control elements may included in the deleted region. In addition, preliminary analysis of two Δ4 transgene-expressing lines show that inducible expression of GFP may be better in αβ T cells relative to the 8 kb construct, while the two different transgenic constructs are expressed at a comparable level in γδ T cells. A high percentage of thymic αβ NKT cells in both Δ4 lines also express the transgene. These results suggest the possibility of an αβ T cell-specific repressive element in the –4.5 kb region which was deleted. A more detailed analysis of these mice is being conducted. Finally, a transgene with an additional 4.8 kb of distal upstream sequence was made to ensure that the DH site mapped to approximately –8 kb would be included. Only 2/5 of the resulting transgene-positive lines expressed GFP well. Those that expressed GFP did not show any clear differences in patterns of spontaneous or inducible expression from the original 8 kb lines.

126. Histone acetylation and DNA-binding proteins of the *IL-2* gene distal regulatory region

Satoko Adachi

The distal upstream regulatory sequences of the IL-2 gene confer position-independent transgene expression, but the mechanism responsible is not known. Because IL-2 is usually not transcribed, even in cells that are programmed to express it upon stimulation, these additional sequences could act to keep chromatin open in a cell type-specific way during periods when the gene is silent, or alternatively, they could simply amplify the number of binding sites available to stimulation-dependent transcription factors, enabling them to open and gain access to previously closed chromatin. A central question is whether the IL-2 locus is associated with open or closed chromatin in potential IL-2 producing cells during periods when the cells are not transcribing the gene. We are focusing on the distal regulatory regions defined by transgenic studies in this lab (see previous abstract and 2001 Annual Report) because it exerts such an influence on whether the gene will be inducible at all.

Acetylation of histones H3 and H4 is associated with open chromatin in many systems. To look the histone acetylation level of the IL-2 gene, chromatin immunoprecipitation assays were performed by using the anti-acetylated histone H3 or H4 antibodies. In EL4 cells, which can express IL-2, histone acetylation was observed from -8.2 kb through the proximal promoter region. In contrast, this was not or very weakly observed in BL3a, SCID adh., and NIH 3T3 cells, which cannot express IL-2. Since the region further upstream than -8.2 kb was negative for histone acetylation in all the cell types, -8.2 kb appears to be an upper limit of histone acetylation of the IL-2 gene. The quantitative analyses by real-time PCR indicated that the stimulation of EL4 cells with PMA and A23187 greatly enhanced the acetylation of -8.2 kb region and -5 kb region which has Btk homology, while most of other regions including the proximal promoter region have not significantly changed. Thus the acetylation of histones

in the IL-2 induction-competent cells is not completely constitutive; there is a possible role for stimulation-dependent mechanisms even in keeping the chromatin open 8 kb upstream from the established promoter/enhancer.

To look for the specific DNA binding proteins that participate in contacts with distal upstream regions, gel shift assay was performed to unstimulated or stimulated EL4, 32D cl.5, and NIH3T3 cells. The probes were designed for the region from -5 kb through -2.3 kb with respect to the promoter, and the length of each probe is about 150 bp. Nine probes out of fifteen detected protein complexes that were specifically observed in stimulated EL4 cells. Some of these probes have predictable NF-κB binding sites at -5 kb, -3.5 kb, and -2.3 kb. The results of chromatin immunoprecipitation assay using the antibodies against NF-κB family proteins (anti-p50, p65, and c-Rel) were also positive for -5 kb and -3.5 kb, indicating that the NF-κB is binding *in vivo* at these sites but is not at -2.3 kb. These results indicate one transcription factor, implicated in proximal promoter/enhancer activity, which is also involved in contacts with the further upstream sequences.

127. T-cell precursors from NOD-*scid/scid* and NOD-*Rag*$^{-/-}$ mice fail to arrest at the β-selection checkpoint

Mary Yui

The β-selection checkpoint is a crucial stage in the development of T cells in the thymus when a pre-T cell must productively rearrange a T-cell receptor (TCR)-β allele or die. Once a TCR-β protein is successfully made, it dimerizes with pre-Tα and forms a pre-TCR signaling complex which initiates dramatic changes in the cell including increased cell survival, proliferation, differentiation, a halt in further TCR-β rearrangement and initiation of TCR-α locus rearrangement. Thymocytes from immunodeficient mouse strains, such as those with the *scid* mutation or *Rag* deficiency are unable to rearrange a TCR and developmentally arrest at this checkpoint, making the thymuses of these mice a rich source of T cell precursors which make up a tiny fraction of a normal thymus. The non-obese diabetic (NOD) mouse is prone to T cell–mediated autoimmune Type 1 diabetes. The immunological defects contributing to autoimmunity in these mice are poorly defined, although some are clearly T-cell intrinsic. To study whether early T-cell development in NOD mice is normal, we have characterized thymocytes from NOD-*scid/scid* and NOD–*Rag*$^{-/-}$ mice. Unlike immunodeficient cells on a normal (C57Bl/6) genetic background, those on the NOD background fail to arrest at the β-selection checkpoint but survive and continue to differentiate even without TCR rearrangement. These cells downregulate CD25, upregulate CD4 and then CD8, at which point the double-positive TCR-negative cells cannot undergo positive selection and die. This breakthrough occurs at approximately 5-6 weeks of age. It appears that the defect may be due to a cell death failure rather than a spontaneous pre-TCR signal because proliferation and restoration of normal thymic numbers does not occur. One interesting possibility is the observation that c-kit, a growth factor receptor gene which is normally downregulated during development, is not downregulated in NOD-scid thymocytes. The presence of an inappropriate growth receptor may enhance survival of these cells. To further investigate the cause of the arrest failure of these cells we are using quantitative real-time RT-PCR to look at expression of apoptotic genes and other genes known to change at β-selection. We hope to be able to determine which cellular pathways are affected by the NOD defect and manipulate those pathways in *in vivo* and *in vitro* systems.

128. Genetic analysis of the NOD-*scid/scid* β-selection checkpoint breakthrough

*Mary Yui, Nkechi Nzerem**

Another approach we are taking to identify the aberrant gene(s) and pathways in the NOD-*scid/scid* that contribute to the β-selection breakthrough defect is to attempt to map the defect to one of the previously mapped diabetogenic loci. There are at least 16 genes in the NOD mouse that are known to contribute to diabetes. Two of these loci are thought to affect mature T-cell or double-positive T-cell survival (on chromosomes 1 and 6, respectively). The specific mutant genes at these different loci and their contribution to autoimmunity are largely unknown. We initiated a genetic cross (NOD-*scid/scid* X B6-*scid/scid*) and found that F1 thymocytes arrest at the β-selection checkpoint, indicating that the NOD trait is recessive. After backcrossing the F1 to NOD-*scid/scid* and phenotyping 48 backcross mice, we found that approximately half of these mice had some significant breakthrough to ISP or DP at 6.5-8 weeks of age. However the distribution of the severity of the breakthrough is not bimodal but rather a continuous distribution, suggesting that it may not be the result of a single simple, fully-penetrant gene, but rather may be due to multiple genes. We are using microsatellite marker (simple sequence repeat polymorphism)-based PCR to compare genotypes for markers that are closely linked to ten of the most important NOD diabetogenic loci to the phenotypes of the 48 backcross mice. So far ten microsatellite markers on six different chromosomes have been monitored in an ongoing analysis.

**MURF fellow*

129. Origins of lymphocyte regulatory networks early in vertebrate evolution

Michele Anderson, Rashmi Pant, Gary Litman, Ellen Rothenberg*

Lymphocytes with rearranging antigen receptors are restricted to the jawed vertebrates, including the cartilaginous fish, whereas jawless vertebrates, such as the lamprey, have cells that morphologically resemble lymphocytes but are lacking the major molecular components of the adaptive immune system. Mammalian lymphocyte development is guided by sets of transcription factors that interact in large regulatory networks. Most of these factors belong to multigene families that arose long before the emergence of the lymphocytes. Therefore, it is likely that they were recruited from more ancient developmental functions such as gut and nervous system development early in vertebrate evolution. We are trying to understand the evolutionary mechanisms that led to the ability of hematopoietic stem cells to develop into the two

major types of lymphocytes, T cells and B cells, which contain related but unique sets of rearranging antigen receptor genes, and whose developmental stages display striking parallels. Many of these parallels may have arisen by the process of gene duplication, which happened on a large-scale 1-2 times, early in the vertebrate lineage, followed by regulatory modifications that sequestered the duplicates into separate expression domains. In order to track these events phylogenetically, we are cloning homologs of transcription factors that are important in lymphocyte development from lower vertebrates and invertebrate deuterostomes, and assessing domain structure, orthology, and amino acid conservation from their sequences. Regulation of the genes is examined using realtime quantitative PCR with specific primers on a spectrum of tissues from each animal.

Our work so far has focused on isolating B-cell transcription factors (EBF, Pax, PU.1) and T-cell transcription factors (GATA-3, Runx) from a cartilaginous fish, *Raja eglanteria*. We have recovered three PU.1 family members, three EBF-1/2/3 family members, two Pax family members, two Runx family members, and one GATA-1/2/3 family member. Expression of skate GATA-3 is closely linked with TCR expression, as it is in mammals. EBF-1 is expressed more widely, but is linked with Ig expression, as expected for a conserved B-cell role. These studies are ongoing, but suggest conservation of many of the developmental networks required for mammalian lymphocyte development throughout the jawed vertebrates. We have also recovered a PU.1 family member and a GATA family member from the lamprey. The lamprey genes appear not to be true orthologs of PU.1 or GATA-3, respectively, and may have arisen from pre-duplication ancestors. Strikingly, both of these genes have conserved DNA binding domains but divergent protein-protein interaction domains. This indicates that both coding regions and regulatory regions underwent modification during recruitment to the lymphocyte lineages. Additional work is underway to define the expression patterns of these lamprey transcription factors, and to isolate additional genes from the lamprey and from amphioxus, a basal chordate, to further define the origins of these transcriptional networks. It is our hope to eventually be able to examine the *cis*-regulatory sequences of these genes using computational comparative genomic analysis as well.

**University of South Florida, Department of Pediatrics/Children's Research Institute*

130. Construction of an arrayed lamprey BAC genomic library for evolutionary analysis of regulatory gene networks

Michele Anderson, David McCauley, Marianne Bronner-Fraser, Ellen Rothenberg

The sea lamprey *Petromyzon marinus* is a member of the basal clade of jawless vertebrates and thus occupies an important phylogenetic position for molecular studies of vertebrate evolution. Of particular interest to the Bronner-Fraser and Rothenberg laboratories are the molecular mechanisms by which vertebrate-specific cell types such as neural crest (Bronner-Fraser) and lymphocytes (Rothenberg) arose. Our two laboratories have been approaching this problem by isolating lamprey homologs of genes known to be important in regulating development of these cell types in higher vertebrates. The lamprey homologs of these genes often exhibit both shared and unique characteristics in terms of structure and expression patterns as compared with other vertebrates. Further studies will require a genomic resource that is currently unavailable. Therefore, we are constructing a genomic BAC library from lamprey DNA that will be arrayed and spotted at high density on nylon filters. The lamprey BAC library will be useful in studies of synteny (conservation of gene linkage), in elucidation of gene number, structure, and genomic organization, and in the isolation of regulatory elements that can be functionally evaluated using cross-species transgenic approaches. These studies will provide insight into the evolutionary changes that lead to new developmental regulatory networks, and thus new cell types.

Publications

Anderson, M.K., Hernandez-Hoyos, G., Dionne, C.J., Arias, A.M., Chen, D. and Rothenberg, E.V. (2002) Definition of regulatory network elements for T-cell development by perturbation analysis with PU.1 and GATA-3. *Dev. Biol.* **246**:103-121.

Anderson, M.K., Weiss, A.H., Hernandez-Hoyos, G., Dionne, C.J. and Rothenberg, E. V. (2002) Constitutive expression of PU.1 in fetal hematopoietic progenitors blocks T-cell development at the pro-T cell stage. *Immunity* **16**:285-296.

Rothenberg, E.V. (2002) T-lineage specification and commitment: A gene regulation perspective. *Semin. Immunol.* 14: In press.

Rothenberg, E.V. and Anderson, M.K. (2002) Elements of transcription factor network design for T-lineage specification. *Dev. Biol.* **246**:29-44.

Rothenberg, E.V. and Davidson, E.H. Regulatory co-options in the evolution of the immune systems of the deuterostomes. In*: Innate Immunity*, R.A.B. Ezekowitz and J.A. Hoffmann, eds. Humana Press, Totowa, N.J. pp. 61-87. In press.

Rothenberg, E.V. and Dionne, C.J. (2002) Lineage plasticity and commitment in T-cell development. *Immunol. Rev.* **187**:96-115.

Rothenberg, E.V., Telfer, J.D. and Yui, M.A. (2002) T-cell developmental biology. In: *Fundamental Immunology,* 5th Edition, W.E. Paul, ed., Lippincott Williams and Wilkins. In press.

Anne P. and Benjamin F. Biaggini Professor of Biological Sciences: Melvin I. Simon
Member of the Professional Staff: Sangdun Choi
Visiting Associates: Emil Bogenmann, Ana Mendez
Senior Research Associate: Iain D.C. Fraser
Postdoctoral Scholars: Bryan Beel, Pamela Eversole-Cire, Lingjie Gu, Sang-kyou Han, Jong-Ik Hwang, Kum-Joo Shin, Tau-Mu Yi
Graduate Student: Cindy Quezada
Research Assistants: Anna Cao, Mi Sook Chang, Sam Couture, Rebecca Hart, Jong Woo Kim, Sun Young Lee, Jamie Liu, Joelle R. Zavzavadjian
Research and Laboratory Staff: Joyce Kato, Santiago Laparra, Josephine Macenka, Valeria Mancino, Blanca Mariona, Mei Wang
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Summary: The major focus of our laboratory continues to be to understand the signal transduction networks that process changes in the concentrations of specific signaling substances in the environments of the cell into instructions that modify cell physiology and gene expression. More than half of the laboratory is engaged in the project involving the Alliance for Cell Signaling (http://www.afcs.org). We are just completing the second year of this large project, which is an attempt to bring to bear a variety of approaches to understand cellular responses to specific ligands that activate cell surface receptors. The focus of the work is on primary B cells and on cardiac myocytes. The first two years of the project has been mostly concerned with building tools to do the analysis. Our laboratory acts as the Center for Molecular Biology for the Alliance. We have made great progress in the last eighteen months in developing platforms for doing transcript analysis using DNA microarrays, generating collaborations with Myriad Genetics, Agilent Technologies, and Isis Corporation. We are coordinating a large-scale study of protein-protein interaction with Myriad Genetics using the yeast two-hybrid system. In addition, we are using antisense and RNAi constructs to study the effects of the elimination of specific gene products on the signal transduction machinery. We have collaborated with Agilent Technologies and designed, built, tested, and applied arrays of 16,000 cDNAs to analyze the effects of 30 different ligands on early-stage B-cell transcription.

In addition to our work with the AfCS, we are continuing studies on the G protein-mediated signaling systems both in the visual system and on the function of specific G proteins and specific orphan G-protein receptors. Some of that work is described in the abstracts below.

Finally, we have continued to work on the bacterial chemotaxis system with a sharp focus on the central signaling complex, which includes the CheA protein. In the past we crystallized this protein and we are currently involved in trying to understand the dynamics involved in its function when complexed to receptor and coupling proteins.

131. Molecular Biology Laboratory of the Alliance for Cellular Signaling: Protein-Protein Interaction (PPI) Project

Joelle R. Zavzavadjian, Sam Couture, Melvin I. Simon, Iain D.C. Fraser

In order to further address the complex task of mapping cellular signaling pathways, the Alliance for Cellular Signaling (AfCS at: http://www.afcs.org) launched, in October 2001, the Protein-Protein Interaction (PPI) project.

The goal of this project is to establish methodology that allows routine isolation of protein complexes in sufficient amounts for analysis by mass spectrometry. In the context of this overall goal, the AfCS Molecular Biology Laboratory at Caltech designs retroviral-based vectors for expression of tagged proteins. A retroviral-based expression system was chosen because the B lymphocyte, one of the model cells chosen by the AfCS for studying signaling pathways, responds poorly to conventional transfection methods. Therefore, transduction of B lymphocytes would allow for higher expression levels of bait protein, thus permitting more efficient isolation of complexes. To date, we have constructed eight retroviral constructs based on the commercially available pMSCV and pLPCX vectors (Clontech). These constructs contain either a Flag-Tev-Myc (FTM) or Tandem Affinity Purification (TAP) tag capable of fusing with the N- or C-terminus of a given protein. The TAP tag is being used in addition to the FTM tag because the protein A and CBP domains of the TAP tag allow for more efficient recovery of low abundance fusion proteins in a complex mixture, and may therefore be more effective with proteins which do not express to high levels. These vectors have been converted into destination vectors that, under Invitrogen's GATEWAY Cloning system, have the ability to efficiently recombine with a gene flanked by the appropriate recombination sites. This strategy thus allows for a high-throughput method of generating expression vectors.

In collaboration with two other AfCS laboratories, the Cell Preparation and Analysis Laboratory and the Protein Chemistry Laboratory, both based at UTSW Medical Center, preliminary results indicate that this approach can yield valuable data to help elucidate signaling pathways. Once results are validated, data will be available at: http://www.afcs.org.

132. Molecular Biology Laboratory of the Alliance for Cellular Signaling: Yeast Two-Hybrid Project

Iain D.C. Fraser, Joelle R. Zavzavadjian, Qingli Mi, Jamie Liu, Mei Wang, Sam Couture, Melvin I. Simon

Identification of protein-protein interactions and the changes in such interactions that result from ligand binding or covalent modification are clearly central to understanding the mechanisms of signal transduction and the establishment of intracellular signaling networks. The Alliance for Cellular Signaling (AfCS at http://www.afcs.org) will utilize high-throughput methods to detect protein-protein interactions between signaling molecules expressed in B cells and cardiomyocytes. In an effort to produce a large body of information on protein-protein interactions in these cells, the AfCS has arranged a collaboration with Myriad Genetics (http://www.myriad.com) to perform yeast two-hybrid screens at the rate of 500 per year.

B cell and cardiomyocyte specific activation domain libraries have been prepared and are currently being screened with baits derived from approximately 100 signaling proteins. Interaction data from approximately 20 proteins is now available (follow "Data" link at http://www.afcs.org). One of early focuses of the AfCS will be to study signaling events that occur upon oligomerization of the B-cell antigen receptor (BCR). Our yeast two-hybrid data identifies several well-established interactions, and also some intriguing novel interactions. Some established interactions include the PI3K p110 alpha catalytic subunit and its regulatory subunit p85 alpha, and the serine/threonine kinase Pak with the small G-protein Rac. Novel interactions between proteins previously implicated in this pathway include PLC gamma with the lipid phosphatase SHIP and the tyrosine kinase Btk with the protein phosphatase calcineurin. Further novel interactions identify proteins not previously implicated downstream of the BCR. The serine/threonine kinase PDK1 binds a member of the cyclic nucleotide phosphodiesterase family, PDE4B3, which may have implications for the cAMP pathway since protein kinase A has been identified as a substrate and binding partner for PDK1. The adapter protein BLNK pulled out BANP, a putative transcription factor that may be involved in cell-cycle regulation and may tie in with BLNKs essential role in B-cell proliferation. Due to the artificial nature of the yeast two-hybrid these interactions require validation in a mammalian cell system to support their physiological significance. However, this data will be valuable, not just to the AfCS but to the scientific community as a whole, to be either proved or disproved by further experimentation.

133. Molecular Biology Laboratory of the Alliance for Cellular Signaling: Serine/Threonine Kinase (STK) Project

Joelle R. Zavzavadjian, Sam Couture, Melvin I. Simon, Iain D.C. Fraser

One of the major tasks of the AfCS Molecular Biology Laboratory at Caltech is construction of full-length mouse cDNAs. During our second year, we have concentrated our efforts on constructing and providing plasmid cDNAs for use in other AfCS laboratories, particularly, the AfCS Microscopy Laboratory at Stanford University. The Serine/Threonine Kinase (STK) project aims to clone 215 STKs fused at either end with CFP or YFP. Since September 2001, we have cloned and fully sequence verified approximately 100 kinases using Invitrogen's GATEWAY Cloning Technology. This system is based on the well-characterized phage-lambda site-specific recombination system allowing the transfer of DNA segments, in a high-throughput format, between different cloning vectors while maintaining orientation and reading frame. This has facilitated the generation of approximately 400 STK expression constructs for microscopical analysis. Expressing these STKs in B lymphocytes, the Microscopy Laboratory uses confocal microscopy to acquire information about subcellular localization of these signaling proteins; and to observe the changes in their location that occur in response to activation of signaling pathways, since such events can be used as readouts of the time-dependence of signaling processes. In the future, cellular imaging, via Fluorescence Resonance Energy Transfer (FRET) and fluorescent biosensors, will play a critical role in mapping components of signaling pathways using various perturbation strategies. In addition, quantifying the flux of information through these pathways provides insight into the chemical and structural basis of cellular localization and translocation. Given the large number of genes to study, the Microscopy Lab is developing and implementing procedures for high-throughput acquisition and analysis of quantitative imaging data. Once results are validated, data will be available at: http://www.afcs.org.

134. Transcript analysis with DNA microarrays

Sangdun Choi, Sun Young Lee, Mi Sook Chang, Anna Cao, Rebecca Hart, Jong Woo Kim, Mei Wang, Melvin I. Simon

We have developed our own DNA microarray protocol using several different platforms. Currently we have inkjet printed 16K cDNA arrays, inkjet printed 16K oligonucleotide arrays, Affymetrix GeneChip system, pin-spotted 16K cDNA arrays and a few others. We have used direct labeling, indirect labeling, and amplification methods. Based on our diverse experience, we have successfully applied these approaches to several of our own research projects and several collaboration projects. One of our main areas of research is centered on ligand screening of B cells in mouse models as a part of Alliance for Cellular Signaling (http://afcs.org). The microarray analysis of the B-lymphocyte single-or double-ligand

screen involves treating the well-characterized B cells with ligand, collecting samples, and using RNA extracted at different times after ligand addition for transcript analysis. The following ligands were already assessed; anti-IgM, BLC, CD40L, IL10, IL4, SDF1, Terbutaline, Bombesin, IFNgamma, IGF-1, TNFalpha, LTB4, NGF, SLC, PGE, fMLP, ELC, S1P, NEB, LPA, BAFF, Dimaprit, LPS, CpG, and 2-MT. Ten more single ligands are being screened. Some of our microarray data can be found in the Caltech microarray lab web site (http://afcs.caltech.edu/afcs) and the display of the entire microarray data together with calcium, cAMP and phosphorylation data are presented in the AfCS database (http://afcs.org).

Our activities also include other collaboration-based projects. These projects include light-induced apoptosis in mouse photoreceptors, the Galpha13 pathway, B-cell receptor pathway, NF-κB pathway, and long-term memory formation.

135. Interaction of $G\alpha_{12}$ with $G\alpha_{13}$ and $G\alpha_q$ signaling pathways

Jennifer L. Gu, Stefan Müller[1]*, Valeria Mancino, Stefan Offermanns[2], Melvin I. Simon*

The G12 subfamily of heterotrimeric G-proteins consists of two members, G_{12} and G_{13}. Gene targeting studies have revealed a role for G_{13} in blood vessel development. Mice lacking the α-subunit of G_{13} die around embryonic day 10 as a result of an angiogenic defect. On the other hand, the physiological role of G_{12} is still unclear. To address this issue, we generated $G\alpha_{12}$-deficient mice. In contrast to the $G\alpha_{13}$-deficient mice, $G\alpha_{12}$-deficient mice are viable, fertile and do not show apparent abnormalities. However, $G\alpha_{12}$ does not seem to be entirely redundant since in the offspring generated from $G\alpha_{12}$+/- $G\alpha_{13}$+/- intercrosses, at least one intact $G\alpha_{12}$ allele is required for the survival of animals with only one $G\alpha_{13}$ allele. In addition, $G\alpha_{12}$ and $G\alpha_{13}$ showed a difference in mediating cell migratory response to lysophosphatidic acid in embryonic fibroblast cells. Furthermore, mice lacking both $G\alpha_{12}$ and $G\alpha_q$ die *in utero* at about E13. These data indicate that the $G\alpha_{12}$-mediated signaling pathway functionally interacts not only with the $G\alpha_{13}$- but also with the $G\alpha_{q/11}$-mediated signaling systems.

[1]*Axxima Pharmaceuticals AG, Am Klopferspitz 19, 82152 Martinsried, Germany*

[2]*Pharmakologisches Institut, Universitat Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany*

**These authors contributed equally to this work*

136. Smooth muscle cell abnormality may contribute to the angiogenic defect in G13-/-mice

Jennifer L. Gu, Yosuke Mukoyama, Shirley Pease, David Anderson, Melvin I. Simon

Vascular morphogenesis is generally classified into two phases for both endothelial cells and smooth muscle cells: vasculogenesis, refers to the differentiation of endothelial cells from hemangioblasts, and subsequent formation of endothelial tubes, and angiogenesis, refers to the subsequent remolding of this primary vasculature into large and small vessels through the sprouting of new blood vessels from pre-existing ones; smooth muscle cells cover endothelial cells during vascular myogenesis, and stabilize vessels during arteriogenesis. Mice deficient for Gα13 gene are observed to have angiogenic defect and die around embryonic day 10. Whole embryo staining with endothelial cell marker PECAM-1 and smooth muscle cell marker ASMA showed normal large vessel structure but defective smaller vascular networks as well as the disappearance of the vascular smooth muscle cells in Gα13 mutant mice. To test the hypothesis that Gα13 is involved in the angiogenic process of endothelial cell sprouting and/or the migratory response of vascular smooth muscle cells to cover the vessel structure, we established embryonic stem cell lines from inner cell mass of Gα13-deficient mice and wild-type mice. At least three different embryonic stem cell lines were derived from each of the G13+/+, G13+/-, and G13-/- genotypes. We further differentiated these embryonic stem cells into vascular smooth muscle cells and endothelial cells *in vitro*, as characterized by several markers such as PECAM1, endoglin, VE-cadherin, CD34 for endothelial cells, and ASMA, desmin, SM-MHC, SM22 for smooth muscle cells. These cells will be tested for their migratory responses to Gα13-coupled GPCR ligands such as LPA, S1P, as well as their cross-interaction with PDGF pathway.

137. RGS6 protein stability may require interaction with the Gβ5 subunit

Pamela Eversole-Cire, Ching-Kang Chen[1], Valeria Mancino, Melvin I. Simon

Regulators of G-protein signaling (RGS) proteins function as GTPase-activating proteins of certain Gα subunits. RGS proteins terminate signaling through the Gα and Gβ/γ subunits by stimulating the intrinsic GTPase activity of the Gα subunit. Presently, more than 20 members of the mammalian RGS protein family have been identified that are grouped into six subfamilies based on structural domains. One subfamily that is comprised of RGS6, RGS7, RGS9, and RGS11 differs from the other RGS proteins in that these proteins have a dishevelled/egl-10/pleckstrin-homology domain (DEP) and a G-protein gamma subunit-like domain (GGL) in addition to the common RGS domain. Although the cellular function of the DEP domain is not yet known, the GGL domain has been shown to interact with the G-protein β5 subunit. This subfamily of RGS proteins and Gβ5 share a similar distribution pattern of their messages in neural tissues suggesting that these proteins may function as a complex in G-protein-mediated signaling in the nervous system.

Previously, it has been demonstrated that mice that lack RGS9 do not have Gβ5 protein even though Gβ5 message is present. Conversely, mice that lack Gβ5 do not have RGS9 nor RGS7 protein but do express RGS4 protein. These results indicate that the RGS proteins that contain a GGL domain may need to interact either during

or immediately after translation with Gβ5 and that this interaction is necessary for maintaining the stability of the complex. It is not yet known if a similar requirement for an interaction between RGS6 and Gβ5 is also necessary for maintaining the RGS6/Gβ5 complex. Therefore, we have recently cloned full-length coding sequences for the murine RGS6 from a brain cDNA library in order to assist in our studies. Multiple forms of murine RGS6 were cloned including a long form and a short form that differ in an 18 amino acid insertion at the C-terminal region of the protein that are similar to previously reported forms cloned for human RGS6. We have generated an antibody to the *c*-terminal region of the protein and have used this to demonstrate that RGS6 protein may also be destabilized in certain neuronal tissues in mice that lack Gβ5.

[1]*Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, UT 84112*

138. MrgA1 and MrgCll are distinctively activated by RF-amide peptides through $G\alpha_{q/11}$

Sang-Kyou Han, Xinzhong Dong, Jong-Ik Hwang, David J. Anderson, Melvin I. Simon

MrgA1 and mrgC11 belong to a recently identified family of orphan G-protein coupled receptors (GPCRs), called mrgs (mas-related genes), they are only expressed in a specific subset of sensory neurons that are known to detect painful stimuli. However, the precise physiological function of Mrgs receptors and their underlying mechanisms of signal transduction are not known. We therefore have screened a series of neuropeptides against human embryonic kidney (HEK) 293 cells that stably express either MrgA1 or MrgC11 in order to identify ligands. Each mrgA1 or mrgC11 specific agonist stimulated dose-dependent increases in intracellular free Ca^{2+} and inositol phosphate production in a pertussis toxin (PTX)-insensitive manner, but failed to alter basal or forskolin-stimulated levels of intracellular cyclic AMP (cAMP). Furthermore, studies using embryonic fibroblasts derived from various G-protein knock-out (KO) mice demonstrated that both the mrgA1 and mrgC11 receptors are coupled to the $G\alpha_{q/11}$ signaling pathway. Screening of neuropeptides identified many surrogate agonists, most of which a common C-terminal –RF(Y)G or –RF (Y) amide motif. Structure-function studies suggest that endogenous ligands of mrgs receptor family are likely to be RF(Y)G and/or RF(Y) amide related peptides and post-processing of these peptides may serve to determine Mrg receptor-ligand specificity. The differences in ligand specificity also suggest functional diversity amongst the Mrg receptors.

139. Functional analysis of the *Thermotoga maritima* CheA histidine kinase

Bryan D. Beel, Melvin I. Simon

Our specific aim is to study the biochemistry of CheA from the hyperthermophilic bacterium *Thermotoga maritima*. CheA is a dimeric histidine kinase that controls the flow of phosphate groups through the signaling pathway controlling chemotaxis in many species of bacteria. This pathway is the best studied of the two-component signal transduction systems common to prokaryotes, and structures of all its proteins have now been solved. CheA is in a cytoplasmic membrane-bound complex with chemoreceptors and the coupling protein CheW; it controls chemotaxis by regulating the flow of phosphate groups through intracellular response regulators. The crystal structure of a portion of CheA from *Thermotoga* was solved in the Simon lab. Based on this structure, we began an investigation into the biochemistry of CheA. We isolated all the chemotaxis genes from *Thermotoga* genomic DNA and inserted them into a set of expression vectors for protein production in *E. coli*. We developed purification protocols for these proteins, expressed, and isolated them in pure form. Initial results show that *Thermotoga* CheA is active at very high temperatures, remains folded, and undergoes surprisingly facile subunit exchange. In collaboration with the Shokat group at UCSF and the Yount group at Washington State, we made preliminary steps toward identifying the characteristics of inhibitors of CheA. Further, we made multiple singly substituted site-directed cysteine mutants of CheA for use in EPR and FRET studies. The current focus of our work involves investigation of the dynamic nature of CheA during signaling. EPR experiments by the Langen group at USC have shown that CheA's active structure contains subtle differences from its crystal structure. We will use EPR to investigate the dynamics of the CheA protein under conditions where its activity is altered by the presence of nucleotides, nucleotide analogues, and other proteins. We will use this same set of cysteine-substituted CheAs in FRET experiments to map out the spatial relationship between CheA, its coupling protein, and the chemoreceptors.

140. Structure-function studies of the histidine kinase, CheA

Cindy M. Quezada, Alexandrine M. Bilwes, Brian R. Crane, Melvin I. Simon

Protein histidine kinases (PHKs) regulate a wide variety of cellular responses in bacteria, fungi, and plants by initiating phosphorelays in response to environmental stimuli. Bacterial chemotaxis, the directed movement towards attractants and away from repellents, uses a two-component signaling system with the autophosphorylating PHK CheA acting as a central element.

The goal of this work is to elucidate the biochemical mechanism of CheA. We have identified an amino acid (E67) in the phosphotransfer domain that is crucial to the phosphorylation reaction. This residue is highly conserved in CheA and also in a family of ATPases, where it is thought to play a key catalytic role. Our lab has determined the structures (ranging from 0.98 Å to 1.2 Å) of the *Thermotoga maritima* phosphotransfer domain and two mutations at position 67. The structures reveal that a mutation as minor as E67Q disrupts a hydrogen bond network we believe to be indispensable to the phosphorylation reaction. The high-resolution structures

of the phosphorylation domain provide a partial atomic view of the CheA active site.

141. Modeling the yeast pheromone response

Tau-Mu Yi, Hiroaki Kitano[1], Melvin I. Simon

Haploid budding yeast cells respond to a peptide pheromone from their partner by undergoing a series of events in preparation for mating. This response is mediated by a heterotrimeric G-protein pathway. Our first goal is to measure quantitatively the dynamics of G-protein activation using a FRET-based system. The alpha-subunit of the G protein (Gα) has been tagged with CFP; the beta/gamma subunits (Gβγ) were tagged with YFP. Association of the heterotrimer resulted in a FRET signal. This signal was lost upon stimulation with pheromone in a dose-dependent fashion. We have measured both the kinetics and dose-response of G-protein activation in wild-type and mutant strains (e.g., sst2Δ). From these data, we have constructed a simple model of the G-protein activation/deactivation cycle. We are using this model to better understand the link between the G-protein cycle and downstream events of the response (e.g., cell-cycle arrest, polarization, and transcriptional activation).

A second project has been to investigate the dynamics underlying polarization in yeast after treatment with pheromone. We have performed a combination of experiments and modeling to understand this process. We have fused green fluorescent protein (GFP) to key proteins in the pathway and integrated these constructs into the yeast genome. The concentrations and intracellular locations of the fusion proteins were determined using quantitative confocal microscopy. Our focus has been on the role of scaffold proteins in amplifying and coordinating the signals involved in polarization. Indeed, one can establish a temporal order of events based on the recruitment of the various scaffold proteins to the polarization site: Gβγ, Ste5p, Bem1p, Cdc12p, Spa2p, and Bni1p. In this manner, we wish to propose a quantitative model dissecting polarization into initiation, establishment, and propagation phases.

Finally, we would like to use our data and models to better understand the gradient-sensing mechanism in yeast. Yeast cells exhibit polarized growth in the direction of the pheromone source (e.g., mating partner). This response is both sensitive and robust. We hope that comparing this system to other chemotactic networks (e.g., bacteria, *Dictyostelium*) can help elucidate general principles of chemotaxis and chemokinesis.

[1]*Kitano Symbiotic Systems Group*

Publications

Bilwes, A.M., Quezada, C.M., Croal, L.R., Crane, B.R. and Simon, M.I. (2001) Nucleotide binding by the histidine kinase CheA. *Nature Struct. Biol.* **8**:353-360.

Borchers, M.T., Justice, P.J., Ansay, T., Mancino, V., McGarry, M.P., Crosby, J., Simon, M.I., Lee N.A. and Lee, J.J. (2002) Gq signaling is required for allergen-induced pulmonary eosinophilia. *J. Immunol.* **168**:3543-3549.

Choi, S., Hao, W., Chen, C.-K. and Simon, M.I. (2001) Gene expression profiles of light-induced apoptosis in arrrestin/rhodopsin kinase deficient mouse retinas. *Proc. Natl. Acad. Sci. USA* **98**:13096-13101.

Dong, X., Han, S.-K. Zylka, M.J., Simon, M.I. and Anderson, D.J. (2001) A diverse family of GPCRs expressed on specific subsets of nociceptive sensory neurons. *Cell* **106**:619-632.

Eversole-Cire, P., Chen, J. and Simon, M.I. (2002) Bax is not the heterodimerization partner necessary for sustained anti-photoreceptor cell death activity of Bcl-2. *Invest. Ophthal. & Vis. Sci.* **43**:1636-1644.

Fitz-Gibbon, S.T., Ladner, H., Kim, U.-J., Stetter, K.O., Simon, M.I. and J. H. Miller. (2002) Genome sequence of the hyperthermophilic crenarchaeon *Pyrobaculum aerophilum*. *Proc. Natl. Acad. Sci. USA* **99**:984-989.

Gu, J.L., Müller, S., Mancino, V., Offermanns S. and Simon, M.I. (2002) Interaction of Gα12 with Gα13 and Gαq signaling pathways. *Proc. Natl. Acad. Sci. USA*. **99**:9352-9357.

Hao, W., Wenzel, A., Obin, M.S., Chen, C.-K., Brill, E., Kransnoperova, N.V., Eversole-Cire, P., Kleyner, Y., Taylor, A., Simon, M.I., Grimm, C., Reme, C.E. and Lem, J. (2002) Evidence for two apoptotic pathways in light-induced retinal degeneration. *Nature Genet*. **32**:254-260.

He, W., Lu, L., Zhang, X., El-Hodiri, H.M., Chen, C-K., Slep, K.C., Simon, M.I., Jamrich, M. and Wensel, T.G. (2000) Modules in the photoreceptor RGS9-1·$G_{\beta 5L}$ GTPase-accelerating protein complex control effector coupling, GTPase acceleration, protein folding, and stability. *J. Biol. Chem.*, Published on-line September 7, 2000 as 10.1074/jbc.M006982200; **275**:37093-37100.

Hurowitz, E.H., Melnyk, J.M., Chen, Y.-J., Kouros-Mehr, H., Simon, M.I. and Shizuya, H. (2000) Genomic characterization of the human heterotrimeric G-protein α, β, and γ subunit genes. *DNA Res.* **7**:111-120.

Kabarowski, J.H.S., Feramisco, J.D., Le, L.Q., Gu, J.L., Luoh, S.-W., Simon, M.I. and Witte, O.N. (2000) Direct genetic demonstration of Ga13 coupling to the orphan G protein-coupled receptor G2A leading to RhoA-dependent actin rearrangement. *Proc. Natl. Acad. Sci. USA* **97**:12109-12114.

Lyubarsky, A.L., Chen, C.-K., Naarendorp, F., Zhang, X., Wensel, T., Simon, M.I. and Pugh, Jr., E.N. (2001) RGS9-1 is required for normal inactivation of mouse cone phototransduction. *Mol. Vis.* **7**:71-78.

Mendez, A., Burns, M.E., Roca, A., Lem, J., Wu, L.-W., Simon, M.I., Baylor, D.A. and Chen, J. (2000) Rapid and reproducible deactivation of rhodopsin requires multiple phosphorylation sites. *Neuron* **28**:153-164.

Miura, M., Watanabe, M., Offermanns, S., Simon, M.I. and Kano, M. (2002) Group I metabotropic glutamate receptor signaling via Gαq/Gαll secures the induction of long-term potentiation in the hippocampal area CA1. *J. Neurosci*. **22**:8379-8390.

Satterthwaite, A.B., Willis, F., Kanchanastit, P., Fruman, D., Cantley, L.C., Helgason, C.D., Humphries, R.K., Lowell, C.A., Simon, M.I., Leitges, M., Tarakhovsky, A., Tedder, T.F., Lesche, R., Wu H. and Witte, O.N. (2000) A sensitized genetic system for the analysis of murine B-lymphocyte signal transduction pathways dependent on Bruton's tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **97**:6687-6692.

Venter, J.C., Adams, M.D., Myers, E.W., P. W. Li, P.W., Mural, R.J. *et al*. (2001) The sequence of the human genome. *Science* **291**:1304-1351.

Yue, C., Ku, C.-Y., Liu, M., Simon, M.I. and Sanborn, B.M. (2000) Molecular mechanism of the inhibition of phospholipase C β3 by protein kinase C. *J. Biol. Chem.* **275**:30220-30225.

Professor of Biology: Paul W. Sternberg
Senior Research Fellow: Jane E. Mendel
Research Fellows: Carol Bastiani, L. René Garcia, Lisa Girard, Bhagwati Gupta, Byung Hwang, Takao Inoué, Nadeem Moghal, Hans-Michael Müller, Gary Schindelman, David Sherwood, Cheryl Van Buskirk, Allyson Whittaker, Xian-Zhong Xu
Graduate Students: Yen Bui, Chieh Chang, Martha Kirouac, Jasper Simon, Hui Yu
Research and Laboratory Staff: Mary Alvarez, John DeModena, Shahla Gharib, Yvonne Hajdu-Cronin, Young-Mee Kim, Gladys Medina, Barbara Perry
WormBase Staff: Juancarlos Chan, Wen Chen, Eimear Kenny, Ranjana Kishore, Raymond Lee, Cecilia Nakamura, Andrei Petcherski, Erich Schwarz, Daniel Wang
Collaborators: Jehoshua Bruck, Melvin Simon, Robert Stirbl, Barbara Wold

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Summary: Our laboratory uses a molecular genetic approach to study basic questions in developmental biology, neurobiology and evolutionary biology: What are the molecular mechanisms of intercellular signaling? How is the fate of a cell specified in response to several intercellular signals? How do genes and neurons control complex behavior? What types of changes in development occur during evolution? How can we use the full genome sequence of an organism? We primarily use the model organism *Caenorhabditis elegans* to study these questions. By understanding several aspects of this one organism in exquisite detail we hope to answer these fundamental biological questions. Our general approach is to use genetics to identify genes controlling the structure and behavior of cells and the whole organism, and to study those genes and their products, and how those genes interact to control development or behavior. In addition, we have a long-standing interest in the evolution of control mechanisms. Our research areas include: signaling by EGF-receptor and its negative regulation as a paradigm for signal transduction; vulval pattern formation and morphogenesis as a paradigm for organogenesis; sinusoidal movement including the role of G proteins Go and Gq; male mating behavior as a paradigm for the genetic control of development; modeling signal transduction; and bioinformatics. Our emphasis has been strongly focused on signal transduction but has more recently expanded to include transcriptional regulation.

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

Mating behavior, with its multiple steps, is arguably the most complex of *C. elegans* behaviors and because it is not essential for reproduction, given the presence of internally self-fertilizing hermaphrodites, is useful to elucidate how genes control behavior. We are studying several aspects of male mating behavior to understand the neuronal circuits that control the behavior and how they are genetically encoded. Most *C. elegans* behaviors rely on the heterotrimeric G proteins Go and Gq, which we have found to act antagonistically. Sinusoidal movement provides an opportunity to study interactions among G-protein signaling pathways as well as more general issues of how genes and cells program behavior. In collaboration with J. Bruck in EAS we have developed a system to observe and extract quantitative data on worm movement; in collaboration with R. Stirbl of JPL we are using worm movement as a way of assessing environmental toxins. Ovulation involves complex regulation of a smooth muscle by a variety of signal transduction pathways, including tyrosine kinases and G protein-coupled pathways; we have analyzed this year regulation of the second messenger inositol 1,4,5 trisphosphate levels. We have also started to analyze a little understood signaling pathway involving spingosine phosphate. In the process of studying G-protein signaling we discovered genes necessary for heat shock response; this year we cloned the *sag-3* locus and found it encodes *C. elegans* heat shock transcription factor.

To address some of the problems of the genomic era, we are working on three types of projects that use computer science approaches. One approach is the

development of tools for "intermediate throughput biology," that is, automation useful for the molecular genetics researcher. A second approach is computer modeling. To understand the pathways and networks underlying development and behavior, it will be necessary to integrate quantitative modeling into our molecular genetic armamentarium. From conventional approaches, genomic approaches and the many "post-genomic" approaches, there is an increasing amount of information that each researcher must have accessible. A new project associated with the laboratory is WormBase, a database of *C. elegans* biology. Through this project, we will provide a service to the *C. elegans* and broader biological communities, as well as work on problems in bioinformatics, such as analysis of *cis*-regulatory sequences, and methods of efficient extraction of information from the literature. As an initial approach to comparative genomics, we have established *C. briggsae* as a developmental genetic system; in terms of molecular sequence divergence, *briggsae* and *elegans* are roughly that of human-mouse, and the *C. briggsae* sequence has just been completed.

142. Mechanisms that establish anchor cell-specific expression of lin-3, *C. elegans* epidermal growth factor (EGF) homolog

Byung Joon Hwang

During *C. elegans* vulval development, the anchor cell (AC), which is a single cell located in the somatic gonad, expresses an EGF-like ligand, LIN-3, that activates an EGF receptor-signaling pathway in vulval precursor cells (VPCs). Studies using laser ablation and mosaic analyses showed that LIN-3 in the AC is essential to induce patterned proliferations of VPCs. To understand how LIN-3 expression is achieved in the AC, we identified a *lin-3* enhancer region that activates its transcription in the AC. The enhancer region contains two E-box elements and one FTZ-F1 nuclear hormone receptor binding site that is mutated in a vulvaless mutant, *lin-3(e1417)*. *In vitro* binding studies and *in vivo* functional assays suggest that trans-acting factors, mammalian E-protein/*Drosophila* Daughterless homolog, HLH-2, and nuclear hormone receptors, activate *lin-3* transcription in the AC. We also show that *lin-3* expresses in the pre-AC/VU cells before AC/VU cell fate determination, and that LIN-12 Notch signaling suppresses LIN-3 expression only in the VU cells, but not in the AC. Thus, LIN-12 Notch signaling pathway interacts with EGF receptor signaling pathway by specifying LIN-3 EGF ligand expression in the AC, which provides an example of how different signaling pathways interact each other.

143. Negative regulation of EGF-receptor signaling

Nadeem Moghal

Negative regulation of receptor tyrosine kinase (RTK)/RAS signaling pathways is important for normal development and the prevention of disease in humans. We have used a genetic screen in *C. elegans* to identify genes that antagonize the activity of activated LET-23, a member of the EGFR family of RTKs. We identified two loss-of-function mutations in *sop-1* that promote the ability of activated LET-23 to induce ectopic vulval fates. SOP-1 is a glutamine-rich protein, most similar to human TRAP230, a component of a transcriptional mediator complex. SOP-1 was previously shown to regulate WNT responses through inhibition of the β-catenin-like protein, BAR-1. We provide evidence that SOP-1 also inhibits RAS-dependent vulval fate specification, and likely regulates the activities of multiple transcription factors during development. Furthermore, we demonstrate that although inhibition of BAR-1-dependent gene expression is dependent on the C-terminal glutamine-rich region, this region is dispensable for inhibition of RAS-dependent cell differentiation. Thus, the glutamine-rich region contributes to promoter selectivity of this class of mediator protein.

Although in the wild, normal animal development occurs under fluctuating environmental conditions, many genes function redundantly in the developmental programs of nematodes, flies, and mice under invariant laboratory conditions, possibly obscuring pathway interactions. The development of the *C. elegans* hermaphrodite vulva is widely used to study EGF receptor-RAS signaling *in vivo*. The first set of mutations that affected vulval development hinted that the environment could modulate its development. Here, we demonstrate that growth in a liquid environment activates a novel pathway for promoting vulval development. This pathway involves activation of the heterotrimeric Gαq protein, EGL-30, which acts via muscle-expressed EGL-19 voltage-gated calcium channels to promote vulval development. We demonstrate that EGL-30 activation and muscle excitation drives vulval development by promoting the activity of BAR-1/β-catenin, which acts parallel to the RAS pathway. Selective modulation of a growth factor pathway by a particular environmental condition provides a context for integrating multiple signaling pathways into a developmental outcome.

144. Genetic analysis of cell invasive behavior

David R. Sherwood

Regulated invasive cellular behavior plays critical roles in trophoblast implantation, organogenesis, wound repair and angiogenesis. Furthermore, it is the loss of control of invasive activity that leads to the development of metastatic cancer, which accounts for the vast majority of cancer deaths. Although critical in both normal development and cancer progression, the regulatory circuits that control invasive cellular behavior remain elusive. We are examining the invasion of the anchor cell (AC) into the vulval epithelium of *C. elegans* to investigate the mechanisms underlying invasive behavior. We have found that the initial steps in building the connection between the separately developing gonad and vulva in *C. elegans* are mediated by the gonadal AC, which crosses the basement membrane separating both tissues, then attaches to and invades between the central vulval cells during the L3 larval stage. AC invasion does

not require neighboring uterine cells, but is stimulated by a secreted signal from the underlying vulval cells. Furthermore, this invasion involves the precise loss of extracellular matrix directly below the AC. To understand the molecular mechanisms that regulate AC attachment and invasion, we have examined numerous known mutants and have discovered that the previously uncloned *evl-5* mutant [Seydoux *et al*. (1993) *Dev. Biol*. **157**:423-436] has a 100% penetrant AC invasion defect. Visualization of AC behavior revealed extensions of lamellipodia and filopodia towards the vulva, but the abrupt flattening of these processes at the basement membrane. In *evl-5* animals the vulva thus produces an invasion cue, but the AC is unable to cross the basement membrane. We cloned *evl-5* and found that it encodes a *C. elegans* homologue of the proto-oncogene *fos* (*Ce-fos*), a component of the AP-1 transcription factor heterodimer. There appears to be only a single *fos* homologue in *C. elegans,* compared with four in vertebrates. We are currently examining the expression pattern of the Ce-FOS protein, and determining the cell(s) in which Ce-FOS functions to regulate AC invasion.

145. Genetic analysis of multivulva mutants in *C. briggsae*

Bhagwati P. Gupta, Jennifer X. Li, Shahla Gharib

To understand the evolution of developmental mechanisms, we are doing a comparative analysis of vulval patterning in *C. elegans* and *C. briggsae*. *C. briggsae* is closely related to *C. elegans* and has identical looking vulval morphology. However, recent studies have indicated subtle differences in the underlying mechanisms of development. The recent completion of *C. briggsae* genome sequence by the *C. elegans* Sequencing Consortium is extremely valuable in identifying the conserved genes between *C. elegans* and *C. briggsae.* We have carried out large-scale EMS mutagenesis and so far isolated seven multivulva (Muv) mutants. Preliminary analysis of the VPC (vulval precursor cell) induction pattern reveals that some of the mutants display phenotypes not previously observed in *C. elegans* Muv mutants. Based on the phenotypes, alleles can be grouped into four different classes. Class I mutants (*sy5216, sy5344* and *sy5392*) have high degree of ectopic induction in P3.p, P4.p and P8.p VPCs. This is somewhat similar to that observed in *lin-1* mutants in *C. elegans. lin-1* encodes an ETS-domain protein that functions as a transcriptional regulator of the VPC cell fates. Class II mutant (represented by a single allele, *sy5342*) has random pattern of VPC induction that is very similar to *lin-31* mutants in *C. elegans. lin-31* encodes a Winged-Helix family of transcription factor that interacts with LIN-1 and functions downstream of LET-60 RAS signaling pathway. Class III mutant (represented by a single allele, *sa993*) has ectopically induced P4.p and P8.p but not the P3.p. Finally, class IV mutants (*sy5353* and *sy5411*) display high frequency of vulval induction in P3.p and P4.p but very low or none in P7.p and P8.p. Current experiments involve genetic mapping and detailed characterization of the vulval induction pattern in mutant animals. These experiments, coupled with molecular analysis, would provide an understanding of the wild-type vulval development and evolutionary changes between the two nematode species.

146. *cis*-Regulatory control of vulval cell-fate markers in *Caenorhabditis elegans*

Martha Kirouac

C. elegans vulval precursor cells (VPCs) undergo up to three rounds of cell division before adopting one of the final vulA, B1, B2, C, D, E and F fates. *egl-17*, *zmp-1*, *cdh-3* are expressed differentially in the developing vulva cells, providing a potential readout for different signaling pathways. To understand how different signaling pathways interact to specify vulval cell types in a precise pattern, we have identified upstream *cis*-regulatory regions that are sufficient for their ability to confer vulval cell type-specific regulation when fused in *cis* to the basal *pes-10* promoter. In the *egl-17* promoter, we have identified a 143 bp region that drives vulC and vulD expression, and a 102 bp region that is sufficient to drive the early expression in presumptive vulE and vulF cells. In the *zmp-1* promoter, we have identified a 300 base pair region that is sufficient to drive expression in vulE, vulA and the anchor cell. In the *cdh-3* promoter, we have identified a 689 bp region sufficient to drive expression in the anchor cell and vulE, vulF, vulD and vulC, a 155 bp region sufficient to drive only anchor cell expression, and a separate 563 bp region that was also sufficient to drive expression in these vulval cells. We have identified the *C. briggsae* homologs of these three genes, and the corresponding control regions, and tested these regions in both *C. elegans* and *C. briggsae*. We conclude that the regions conserved in *C. elegans* and *C. briggsae* upstream of *egl-17*, *zmp-1*, *cdh-3* promote expression in vulval cells. We also conclude that although these *cis*-regulatory elements promote cell specificity in gene regulation, they probably do so by using distinct transcription factors.

147. *lin-17, lin-18* and patterning of the P7.p lineage

Takao Inoue, Rashmi Deshpande, Russell Hill*, Paul W. Sternberg*

We are interested in patterning of the 2° (secondary) vulval lineages P5.p and P7.p. In the wild type, P5.p and P7.p produce stereotyped ABCD and DCBA patterns respectively. In *lin-17* and *lin-18* mutants, the polarity of the P7.p lineage becomes altered. We examined the P7.p lineage in *lin-17* (encoding Frizzled Wnt receptor), *lin-18* (encoding RYK receptor tyrosine kinase-related protein; W. Katz and Paul W. Sternberg), and double mutant using POP-1 (TCF/LEF) antibody staining and cell fate markers *ceh-2::yfp* and *cdh-3::cfp*. In the wild type, POP-1 is expressed in an asymmetric pattern with posterior daughters of P7.p and P7.px expressing a higher level than their respective sisters. In the *lin-17* mutant, the asymmetry among P7.p daughters is often reversed but the asymmetry among P7.px daughters is not strongly affected (R. Hill and J.R. Priess, pers.

comm.). We found that in the *lin-18* mutant, as in the *lin-17* mutant, the asymmetry is often reversed among P7.p daughters but not P7.px daughters. We also found that in the double mutant, the asymmetry is reversed in both P7.p daughters and P7.px daughters. In the mid-L4 stage, *ceh-2::yfp* labels vulB cells (P7.ppax) and *cdh-3::cfp* labels vulC and vulD (P7.paxx). vulA cells of the 2° lineage can be distinguished by non-expression of either markers and adherence to the ventral cuticle in the mid-L4 stage. We found that in *lin-17* and *lin-18* mutants, P7.pap (presumptive vulC) cells were transformed to the vulA fate. In the double mutant, P7.paa (presumptive vulD) cells were transformed to the vulA fate. These results are consistent with reversals of POP-1 expression patterns, and indicate that the lineage that had high POP-1 levels in both P7.px and P7.pxx stages correlate with the cells that adopt the vulA fate. The mechanism by which Ryk receptor signals is not known. The extracellular domain of LIN-18 and Ryk shows homology to Wnt binding protein WIF-1, suggesting a possible mechanism as an alternative Wnt receptor or a Wnt co-receptor. Since a probable *lin-17* null mutation is enhanced by the *lin-18* mutation, LIN-18 can signal independent of LIN-17. Furthermore, a *lin-18::gfp* fusion construct lacking the kinase domain can rescue the *lin-18* mutant, suggesting that LIN-18 functions differently than receptor tyrosine kinases activated by phosphorylation. It is also possible that *lin-18* functions in a separate non-Wnt pathway also involved in P7.p polarity.

**Ohio State University*

148. Male mating behavior

Allyson Whittaker

C. elegans male mating behavior can be organized into at least six steps: response to the hermaphrodite, backing, turning, vulval location, spicule insertion and sperm transfer. Although the signals that trigger these steps are unknown they are likely to include chemosensory, mechanosensory and proprioceptive cues. Male mating provides a basis for understanding how multiple sensory inputs can be processed to coordinate a series of behaviors. A complete understanding of this process will first require knowledge of the regulation of each individual step of mating. One of our foci is on the earliest steps of mating, response, backing and turning.

We have carried out an F2 clonal screen to isolate mutations that disrupt genes required for mating behavior. EMS mutagenized *plg-1*; *him-5* lines were first assayed for their ability to form a copulatory plug over the vulva of hermaphrodite siblings. The mating behavior of lines consistently failing to form a copulatory plug was then analyzed in depth to determine if any of the steps of mating were disrupted. Of 14,000 F2 lines screened approximately 5.9% failed to show copulatory plugs. Within this group were lines that disrupted subsets of each of the steps of mating. We chose a group of six defective lines based on the high penetrance and consistency of their phenotypes for further analysis. No mutations were isolated that only effected response, backing or turning individually. This could suggest redundancy of pathways controlling the steps of mating and/or that the screening procedure disfavored isolation of step-specific mutants. Three mutant lines show defects in response and vulval location behavior. Two mutant lines show defects in response, turning and vulval location, and one shows defects in response and turning. The fact that we have isolated mutations disrupting different subsets of behaviors may provide us with insight into the similarities and differences in the regulation of the steps of mating behavior. Further characterization and mapping of these mutations is in progress.

The phenotypes in two of the lines with response and vulval location defects result from a mutation in a single gene and the mutations are recessive. Complementation tests and initial mapping experiments indicated that the two mutations were likely to be alleles of *pkd-2*, a gene found previously in our lab to be necessary for response and vulval location (Barr *et. al,* 2001). *pkd-2* encodes the *C. elegans* homolog to one of the two major genes disrupted in human autosomal dominant polycystic kidney disease. Sequence analysis showed that in both lines there is a G to A change resulting in a glycine to arginine change in amino acid 565. This amino acid is present in a predicted transmembrane domain. Further analysis of this mutant line may provide insight into the function of this domain.

The mutation *cod-5* was previously isolated in our lab in a screen for lines with reduced mating efficiency. We found that in addition to having strong turning defects, *cod-5* mutants are also defective in response and vulval location. Cloning of *cod-5* is in progress.

149. Hermaphrodite-derived, mate-finding cue implicated in *C. elegans*

Jasper M. Simon

When males of *C. elegans* come into association with their hermaphroditic counterparts they cease foraging behavior and begin to mate. We developed several assays to demonstrate that a soluble cue is correlated with this process. This cue is sexually dimorphic, given off only by the hermaphrodite and eliciting a response only in the male. From our studies we suggest a form of kinesis that works by attracting males to their mating partners from a distance and functions, once males arrive, in holding attracted males in close proximity. We have explored both the variability of this cue between geographically diverse *C. elegans* isolates as well as the extent of this cue in species in and outside the genus *Caenorhabditis*. Ciliated sensory neurons are required for this response. The polycystins PKD-2 and LOV-1 are not. Vulvaless hermaphrodites still make the cue.

150. Specification of the male sensory neuron HOB

Hui Yu

The hook sensory neuron HOB mediates vulva location behavior during *Caenorhabditis elegans* male mating. We screened for mutations that affect HOB gene expression and isolated an allele of zinc finger transcription factor *egl-46.* Mutations in *egl-46* are defective in vulva location, indicating a disruption of HOB function. Establishment of primary neuronal fate of HOB, cell position and morphology, and dendritic process to the male hook are normal in the *egl-46(-)* males. EGL-46 regulates HOB-specific expression of polycystins and several other genes, but is not required for expression of general cilium structure genes in the HOB neuron. By contrast, RFX-like transcription factor *daf-19* is not only an upstream key regulator in general cilium formation pathway, but also affects the expression of downstream targets of EGL-46-regulated program in a non-HOB-specific manner. Therefore, *egl-46* regulates a cell-specific program to define sensory specificity for HOB neuronal function. This program might be dependant upon execution of the ciliogenic pathway.

151. Spicule protraction and an ERG-like potassium channel

L. Rene Garcia

During mating, *C. elegans* males must regulate distinct muscle contractile behaviors to insert their spicules into the hermaphrodite vulva. To understand how genes regulate motor programs during a complex behavior, we isolated EMS-induced mutations that cause males to execute spicule insertion behavior inappropriately. One of the mutants, *sy557*, contains two missense mutations in the *C. elegans* delayed rectifier K+ channel *erg*-homolog *unc-103*. This allele of *unc-103* acts as a dominant negative and may also interfere with other proteins. We isolated additional loss-of-function *(lf)* mutations in *C. elegans unc-103(erg),* and found that reducing this K+ channel's activity leads to premature protraction of the male copulatory spicules prior to mating behavior, and to inappropriate prolonged muscle contractions within sequences of rapid periodic protractor contractions during mating. In humans, mutations in *erg* cause cardiac arrhythmias that can result in sudden heart seizures. Our mutant alleles induce similar types of defects suggesting that the functional role of *unc-103* and human *erg* is conserved. *unc-103(lf)*-induced spontaneous spicule protraction requires input from both the SPC motor neuron and the non-essential protractor auxiliary muscle, both of which connect to the protractor muscles. Mutations that lower acetylcholine production, heterotrimeric Gαq signaling, and calcium signaling through L-type voltage gated calcium channel reduces *unc-103(lf)*-induced spontaneous protraction; suggesting that the UNC-103 ERG-like K+ channel helps keep the spicule protractor muscles inactive from constitutive low levels of cholinergic signaling during periods between matings. *unc-103(lf)* males that do not spontaneously protract their spicules show normal mating behavior up to the spicule insertion step. During this step, instead of sustaining a rhythmic prodding of the vulva slit with their spicules, mutant males erratically extend their spicules completely, depressing the vulval cuticle rather than penetrating it. In contrast, wild-type males fully protract their spicules only when the spicule tips successfully breach the vulval barrier. Thus the UNC-103 ERG-like K+ channel also regulates the proper timing of complete spicule protraction during mating behavior.

152. Sperm transfer during *C. elegans* male mating behavior

Gary Schindelman, Shahla Gharib

C. elegans male mating behavior involves the proper execution of a series of sub-behaviors culminating in the transfer of sperm to the hermaphrodite [Liu, K.S. and Sternberg, P.W. (1995) *Neuron* **14**:79-89]. These sub-behaviors are: response to hermaphrodite, backing, turning, vulval location, spicule insertion and sperm transfer. We are analyzing the genetic control of this stereotyped behavior as it may provide insight into sensory perception and nervous system function.

One of our foci has been on the sperm transfer sub-behavior. Preliminary observation of wild-type sperm transfer has defined four steps in this process: initiation, release, continuation and cessation of transfer. To begin to answer fundamental questions about how sperm transfer is regulated, we are currently doing a genetic screen designed to isolate males defective for this process.

Using an F2 clonal screening approach, 14,000 EMS mutagenized *plg-1(2001d) him-5(e1490)* F3 populations were scored for plugs. Plates that contain no hermaphrodites with mating plugs indicate a possible defect in one or more of the above-mentioned sub-behaviors. These plug-less lines were further characterized and screened for males defective for sperm transfer. Thus far our screening has produced one mutant defective in the initiation step of sperm transfer *(sy671)* and one mutant defective for both initiation and sperm transfer continuation *(sy672)*. These isolated mutants appear morphologically normal.

In order to characterize *sy671* at the molecular level, we are using positional mapping techniques to clone this gene. Molecular and phenotypic markers have been used to place *sy671* in a five-cosmid interval on the X chromosome. The sperm transfer defect in *sy671* was rescued through injection of one of these cosmids and further delineation of the locus is in progress.

153. Characterization of $G\alpha_q$-mediated signaling in *C. elegans*

Carol A. Bastiani, Wen J. Chen, Shahla Gharib, Melvin I. Simon, Paul W. Sternberg

We are using *C. elegans* as a model system to characterize $G\alpha_q$ signaling pathways. *egl-30* encodes the single *C. elegans* homologue of vertebrate $G\alpha_q$ family members. We analyzed the expression pattern of EGL-30 and found that it is broadly expressed, but it is most highly expressed in the nervous system and in pharyngeal muscle.

We isolated dominant, gain-of-function alleles of *egl-30* as intragenic revertants of an *egl-30* reduction-of-function mutation. Using these gain-of-function mutants, we examined the site and mode of action of EGL-30. *egl-30* functions in both the nervous system and in the vulval muscles to regulate egg-laying behavior. Genetic epistasis with mutations that eliminate detectable levels of serotonin reveal that *egl-30* is directly linked to serotonin signaling, and is likely directly coupled to a 5-HT_2 receptor in vulval muscle. We examined genetic interactions with mutations in the gene that encodes the single *C. elegans* homologue of PLCβ, and with mutations in genes that encode molecules downstream of PLCβ. We conclude that PLCβ functions in parallel with *egl-30* with respect to egg laying, or is not the major effector of EGL-30. In contrast, PLCβ-mediated signaling is likely downstream of EGL-30 with respect to pharyngeal-pumping behavior.

Our data indicate that there are multiple signaling pathways downstream of EGL-30, and that different pathways predominate with respect to the regulation of different behaviors. We believe that these results are likely to contribute to the study of vertebrate $G\alpha_q$ signaling since murine $G\alpha_q$, when expressed in *C. elegans*, can also bypass the requirement for PLCβ with respect to egg-laying behavior. We are currently in the process of mapping extragenic suppressors of an *egl-30* reduction-of-function allele that are expected to define other regulators or downstream components of EGL-30-mediated signaling.

EGL-30 and GOA-1 ($G\alpha_o$) regulate diverse behaviors in *C. elegans* in an antagonistic fashion. Our genetic studies indicate a role for RGS proteins as a regulatory link between the EGL-30 and GOA-1 signaling pathways. To date, functional assays in COS-7 cells as well as coimmunoprecipitation assays in HEK293T cells, has confirmed that the two *C. elegans* RGS7 homologues can interact directly with the indicated Gα subunits and that GOA-1-mediated signaling can negatively regulate the EGL-30 pathway. We are further exploring these interactions with respect to the regulatory mechanisms by which these interactions are modulated.

154. Characterization of TRPC channels in *C. elegans*

X.Z. Shawn Xu

The *trp* genes encode a superfamily of cation channels conserved from worms to humans, comprising three major subfamilies, TRP-Canonical (TRPC), TRP-Vanilloid (TRPV) and TRP-Melastatin (TRPM). The canonical TRP (TRPC) channels possess both ankyrin repeats and a TRP domain, and are most closely related to the *Drosophila* TRP and TRPL, the founding members of the TRP superfamily. In contrast, the TRPV and TRPM channels lack either the TRP domain or the ankyrin repeats and are therefore more distantly related to the *Drosophila* TRP and TRPL. While the biological functions and gating mechanisms of the TRPV and TRPM channels are emerging, little is known about the TRPC channels. Although it has been well established that the *Drosophila* TRP and TRPL form the light-sensitive channels in photoreceptor cells, their TRPC homologues in other organisms do not seem to be required in visual transduction. This suggests that TRP and TRPL may have been functionally dedicated to fly vision during evolution and their TRPC counterparts in other organisms may play currently unknown functions. Furthermore, the signaling pathways leading to the activation of the TRPC channels also remain obscure. To address these questions, we have begun to use worms as a model system. The *C. elegans* genome encodes three TRPC genes, *trp-1*, *trp-2* and *trp-3*. We have generated deletion mutants of all the three TRPC genes, each with two independent alleles. Thus, *C. elegans* represents the first organism, in which a complete set of TRPC mutants, have been isolated. *trp-1* and *trp-2* are co-expressed in several classes of excitable cells, whereas *trp-3* is enriched in the sperm cells. We are currently characterizing the mutant phenotypes.

155. Regulation of alternative splicing in *C. elegans*

Cheryl L. Van Buskirk

An estimated 60% of human genes produce multiple splice forms. Despite the prevalence of alternative splicing, very little is known about the regulation of this process. The aim of my work is to isolate factors that regulate alternative splicing, as a starting point for understanding the role of this process in development and in human disease. I am focusing on the splicing regulation of *lin-3* and *ced-4*, genes that are involved in cell-cell signaling and cell death, respectively.

A single EGF receptor ligand, LIN-3, is used for several inductive events during *C. elegans* vulval development, and it is unclear how these multiple signaling events are insulated from each other. *lin-3* cDNAs have been isolated that encode alternate forms of the ligand that may possess different cleavage properties and hence signaling ranges. I aim to isolate factors that regulate the alternative splicing of *lin-3* through a genetic screen that utilizes a LIN-3: diptheria toxin reporter causing isoform-specific cell death. By screening for suppression of this phenotype, I will isolate regulators of *lin-3* alternative splicing. I will then characterize the activity of these splicing regulators and their effects on *lin-3* signaling events, as well as any other effects on development.

The *ced-4* gene of *C. elegans*, homologous to human Apaf-1, is essential for programmed cell death. Interestingly, the *ced-4* gene encodes two alternative splice forms, the rarer of which appears to oppose the function of the predominant isoform, preventing cell death. Hence the balance between these two isoforms may determine whether a cell lives or dies. However, it is now known how this balance is normally regulated. I propose to isolate factors that regulate this splicing decision by screening for mutations that suppress the cell death phenotype caused by ectopic expression of *ced-4*. Characterization of the function of these splicing regulators and determination of their specificity will

contribute to our understanding of alternative splicing, and to our understanding of cell death regulation.

156. WormBase: A database for *C. elegans* biology and genomics

Tamberlyn Bieri[2], Darin Blasier[2], Keith Bradnam[3], Juancarlos Chan, Chao-Kung Chen[3], Wen Chen, Fiona Cunningham[1], Richard Durbin[3], Todd Harris[1], Eimear Kenny, Ranjana Kishore, Dan Lawson[3], Raymond Lee, Hans-Michael Müller, Andrei Petcherski, Anthony Rogers[3], Erich Schwarz, John Spieth[2], Lincoln Stein[1], Gudmundur Thorisson[1], Paul W. Sternberg

WormBase (http://www.wormbase.org/) is the successor to the *C. elegans* genomic database ACeDB. Its two fundamental goals are to make *C. elegans* database more accessible through the web, and to expand the database from gene structures to gene functions, networks, cellular expression patterns, and biological processes. A central element of WormBase is Ph.D.-level curation of the literature and of large-scale datasets. Other goals of WormBase include: improving the accuracy of gene structures through literature curation and reanalysis of ESTs; general improvement of the web interface through direct user feedback; an up-to-date user guide; a systematic checklist of literature curation topics (gene function and structure, transgene composition and gene expression pattern, cellular function) that allows rapid dissection of literature into specialized area of curation expertise; annotation of protein-coding sequences gene ontology, conserved orthologous groups, and Interpro motifs; construction of a broadly usable structural vocabulary for detailed stages of embryonic and post-embryonic development; and incorporating several large-scale data sets (SNPs, mass RNAi phenotypes, and neural wiring diagrams) into WormBase. Presently, we have curated 946 *C. elegans* papers; annotated 1776 gene expression patterns (100% of all expression patterns); imported phenotypes of 7227 genes inactivated by RNAi; annotated 1544 genes, and computationally-mapped gene ontology (GO) descriptions onto 6645 genes. We have also begun manual annotation of *C. elegans* genes to GO terms with 50 annotated genes. Our current work includes continuing to curate the *C. elegans* literature, streamlining the curation process, devising several computational tools to automate curation (Automated Information Extraction from a Literature Database, Hans-Michael Müller, Eimear E. Kenny, and Paul W. Sternberg), and systematic extension of GO to all *C. elegans* genes.

[1]*Cold Spring Harbor Laboratory*
[2]*Washington University at St. Louis, MO*
[3]*Wellcome Trust Sanger Institute*

157. Automated information extraction from a literature database

Hans-Michael Müller, Eimear E. Kenny, Paul W. Sternberg

A major challenge facing post-genomic biomedical science is the biological annotation of genomic sequences. While many biological facts and observations are being derived from large-scale, high-throughput post-genomic projects such as DNA-expression microarrays, proteomics and structural genomics, most information is still obtained from individual papers. Manual extraction of information from scientific papers is expensive, tedious and slow. We therefore seek to design a web-based system that aids the curator in retrieving and extracting information. While an automated system is unlikely to completely replace professional curation, the efficiency of curation of the rapidly growing literature could be increased tremendously.

We have started to devise an information extraction system, which consists of several elements: a first, preprocessing unit prepares and formats plain text files from the corpus of currently 1880 pdf-files of *C. elegans* articles. The text is then tagged semantically according to an ontology we have developed. This ontology contains 39 categories. They can be summarized as classes of biological interest (such as gene, phenotype and cell), of actions, facts or circumstances that relate two (biological) entities or describe one (such as physical association, regulation, and effect), as well as other (auxiliary) classes that are useful for the information extraction process. The ontology also includes all terms from the Gene Ontology (GO) Consortium. The semantically marked-up text is presented in XML format, making it available to XML-processing software tools. The marked-up text will then be used for the fact-extraction of pre-specified types of relationships, events or entities, such as gene-gene interactions or GO-gene associations. The basis for this process is the fact that semantic tags form particular patterns in a sentence because of the relatively rigid structure of the English language. A user interface will then allow the curator to formulate queries in a variety of ways: simple keyword searches, searches for a set of occurrences of categories in a sentence as well as the extraction of specified facts. The curator will be able to download paragraphs and whole articles to verify the returned output, and prepare the extracted facts for further processing and porting to other databases. The project currently focuses on *C. elegans* literature; however, an expansion to the literature of other model organisms should be straightforward.

Publications

Barr, M.M., DeModena, J., Braun, D., Nguyen, C.Q., Hall, D.H. and Sternberg, P.W. (2001) The *Caenorhabditis elegans* autosomal dominant polycystic kidney disease gene homologs *lov-1* and *pkd-2* act in the same pathway. *Curr. Biol*. **11**:1341-1346.

Bui, Y. and Sternberg, P. W. (2002) *C. elegans* inositol 5-phosphatase homologue negatively regulates inositol 1,4,5-triphosphate signaling in ovulation. *Mol. Biol. Cell* **13**:1641-1651.

Garcia, L.R., Mehta, P. and Sternberg, P.W. (2001) Regulation of distinct muscle behaviors controls the *C. elegans* male's copulatory spicules during mating. *Cell* **107**:777-788.

Gupta, B.P. and Sternberg, P.W. (2002) Tissue-specific regulation of the LIM homeobox gene *lin-11* during development of the *C. elegans* egg-laying system. Dev. Biol. **247**:102-115.

Levchenko, A., Bruck, J. and Sternberg, P.W. (2001) Combination of biphasic response regulation and positive feedback as a general regulatory mechanism in homeostasis and signal transduction. In: "Foundations of Systems Biology," H. Kitano, ed., Cambridge, Massachusetts, M.I.T. Press, pp. 263-278.

Schwarz, E.M., Stein, L.D. and Sternberg, P.W. (2002) *Caenorhabditis elegans* databases. *Curr. Genomics* **3**:111-119.

Simon, J. and Sternberg, P.W. (2002) Evidence of a mate-finding cue in the hermaphrodite nematode *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **99**:1598-1603.

Sternberg, P. and Kemphues, K. (2002) The 2001 Genetics Society of America Medal: H. Robert Horvitz. *Genetic*s **160**:369-370.

Howard and Gwen Smits Professor of Cell Biology: Alexander Varshavsky
Senior Research Fellow: Yong Tae Kwon
Postdoctoral Scholars: Christopher Brower, Rong-Gui (Cory) Hu, Hai Rao, Jun Sheng, Takafumi Tasaki, Zanxian Xia, Jianmin Zhou
Research and Laboratory Staff: Jee-Young An, Janet Dyste, Josephine Macenka, Jai Wha Seo

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Summary: Our main subject is the ubiquitin (Ub) system. In the 1980's, my colleagues and I produced the first evidence that Ub conjugation is required for protein degradation in living cells, discovered the first physiological functions of the Ub-dependent proteolysis (in the cell cycle, DNA repair, protein synthesis and stress responses), deciphered the first degradation signals in short-lived proteins, and identified critical mechanistic attributes of the Ub system, particularly the multi-Ub chain and subunit selectivity of protein degradation. Our current work continues to focus on Ub-dependent circuits.

The effect of an intracellular protein on the rest of the cell depends on the protein's concentration. The latter is determined by the rate of synthesis and/or import of the protein in relation to the rates of its degradation, inactivation by other means, or export from the compartment. The *in vivo* half-lives of intracellular proteins range from a few seconds to many days. Over the last decade, a vast number of biological circuits were shown to contain either constitutively or conditionally short-lived regulators. Damaged or otherwise abnormal proteins tend to be short-lived as well. The metabolic instability of a regulator provides a way to generate its spatial gradients and allows for rapid adjustments of its concentration (or subunit composition) through changes in the rate of its synthesis or degradation.

Ubiquitin (Ub) is a 76-residue protein that exists in cells either free or conjugated to many other proteins. Degradation of intracellular proteins by the Ub-proteasome system is an essential part of a vast multitude of processes that include the control of cell growth, division, differentiation, signal transduction, responses to stress, and thus a broad range of metacellular, organismal processes as well, from embryonic development to the immunity and functions of the nervous system. Ub-dependent proteolysis involves the "marking" of a substrate through covalent conjugation of Ub to a substrate's internal Lys residue. Ub conjugation is mediated by the E1-E2-E3 enzymatic cascade. E1, the ATP-dependent Ub-activating enzyme, forms a thioester bond between the C-terminal Gly of Ub and a specific Cys residue of E1. In the second step, activated Ub is transesterified to a Cys residue of a Ub-conjugating (E2) enzyme. Thereafter a complex of E2 and another enzyme, E3, conjugates Ub to a Lys residue of a substrate. The functions of E3 include the recognition of a substrate's degradation signal (degron). The numerous proteolytic pathways of the Ub system have in common their dependence on Ub conjugation and the 26S proteasome (which processively degrades Ub-protein conjugates), and differ largely through their utilization of distinct E2-E3 complexes. Specific E3s recognize (bind to) specific degrons of their substrates. The diversity of E3s and E2s underlies the enormous range of substrates that are recognized and destroyed by the Ub system, in ways that are regulated both temporally and spatially.

One pathway of the Ub system is the N-end rule pathway (Fig. 1). The N-end rule, which relates to the *in vivo* half-life of a protein, to the identity of its N-terminal residue, was discovered by this laboratory in 1986, in experiments that explored the fate of a fusion between Ub and a reporter protein such as *E. coli* β-galactosidase (βgal) in the yeast (fungus) *S. cerevisiae*. In eukaryotes, Ub-X-βgal is cleaved, cotranslationally or nearly so, by deubiquitylating enzymes (DUBs) at the Ub-βgal junction. This cleavage takes place regardless of the identity of the residue X, proline being the single exception. By allowing a bypass of the normal N-terminal processing of a newly formed protein, this result yielded an *in vivo* method (the Ub fusion technique) for generating different residues at the N-termini of otherwise identical proteins, a technical advance that led to the finding of the N-end rule. The *in vivo* half-lives of resulting X-βgal proteins were shown to range from ~2 min (e.g., Arg-βgal or Leu-βgal) to longer than 20 hr (e.g., Met-βgal or Gly-βgal), depending on the identity of their N-terminal residue (Fig. 1). The N-end rule pathway is present in all organisms examined, from mammals and plants to fungi and prokaryotes. The N-end rule-based degradation signal, called the N-degron, consists of a destabilizing N-terminal residue and an internal lysine (or lysines) of a substrate, the Lys residue being the site of formation of a substrate-linked multi-Ub chain. The ubiquitylated substrate is degraded by the 26S proteasome.

The functional and mechanistic study of the N-end rule pathway in yeast and mammals is a major theme of our current work.

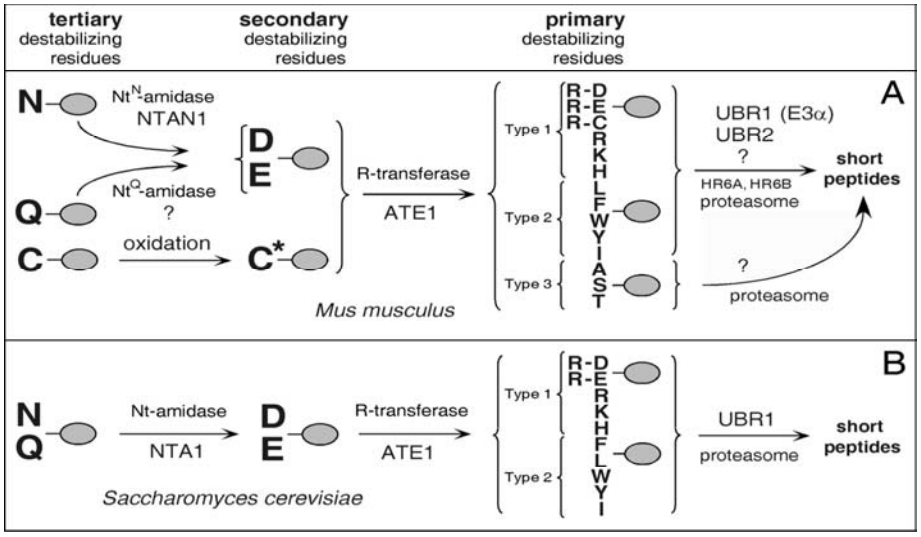

Fig. 1. The N-end rule pathway in mammals (**A**) and yeast (**B**).

158. An essential role of N-terminal arginylation in cardiovascular development

Yong Tae Kwon, Anna Kashina, Rong-Gui Hu, Jee Young An, Jai Wha Seo, Ilia Davydov[1], Alexander Varshavsky

The N-end rule has a hierarchic structure. Specifically, N-terminal Asn and Gln are tertiary destabilizing residues in that they function through their deamidation, by N-terminal amidohydrolases, to yield the secondary destabilizing residues Asp and Glu, whose activity requires their conjugation, by *ATE1*-encoded Arg-tRNA-protein transferases (R-transferases), to Arg, one of the primary destabilizing residues. The latter are recognized by the Ub ligases (E3 enzymes) of the N-end rule pathway. In mammals, the set of destabilizing residues that function through their arginylation includes not only Asp and Glu but also Cys, which is a stabilizing (non-arginylated) residue in the yeast *S. cerevisiae*.

The two characterized species of mammalian Arg-tRNA-protein transferases (R-transferases), ATE1-1 and ATE1-2, are produced through alternative splicing of *ATE1* pre-mRNA (Kwon *et al*., 1999). The ratio of *ATE1-1* to *ATE1-2* mRNA varies greatly among the mouse tissues: it is ~0.1 in the skeletal muscle, ~0.25 in the spleen, ~3.3 in the liver and brain, and ~10 in the testis, suggesting that the two R-transferases are functionally distinct. However, the substrate specificities of ATE1-1 and ATE1-2 are similar to that of the *ATE1*-encoded R-transferase of *S. cerevisiae*, in that they can arginylate N-terminal Asp and Glu, but cannot arginylate N-terminal Cys (Kwon *et al*., 1999). This raises the question of how N-terminal Cys is arginylated in mammalian cells. To address this issue and the physiological functions of arginylation, we constructed *ATE1(-/-)* mouse strains (Kwon *et al*., 2002). It was found that *ATE1(-/-)* cells are incapable of arginylating all three of the secondary destabilizing N-terminal residues, Asp, Glu and Cys, raising the possibility that N-terminal Cys may undergo an enzymatic modification that converts it into a substrate of ATE1. Through biochemical and mass spectrometric analyses, we showed that N-terminal Cys , in contrast to N-terminal Asp and Glu, is oxidized prior to its arginylation by R-transferase, suggesting that the arginylation branch of the N-end rule pathway functions as an oxygen sensor. *ATE1(-/-)* embryos die *in utero* around day E15, primarily from hemorrhages. *ATE1(-/-)* embryos exhibit both defective cardiogenesis and defective angiogenic remodeling of the early vascular plexus. Thus, *ATE1* is required for cardiovascular development, a new set of functions of the N-end rule pathway.

[1]*Present address: IGEN International, Inc., Gaithersburg, MD 20877*

References

Gonda, D.K., Bachmair, A., Wünning, I., Tobias, J.W., Lane, W.S. and Varshavsky, A. (1989) *J. Biol. Chem.* **264**:16700-16712.

Balzi, E., Choder, M., Chen, W., Varshavsky, A. and Goffeau, A. (1990) *J. Biol. Chem.* **265**:7464-7471.

Kwon, Y.T., Kashina, A.S. and Varshavsky, A. (1999) *Mol. Cell. Biol*. **19**:182-193.

Kwon, Y.T., Kashina, A.S., An, J.Y., Seo, J.W., Hu, C. and Varshavsky, A. (2002) *Science* **297**:96-99.

159. Mechanistic and functional analyses of N-terminal arginylation

Christopher Brower, Rong-Gui Hu, Jun Sheng, Jianmin Zhou, Yong Tae Kwon, Alexander Varshavsky

Several functional and mechanistic studies of the N-terminal arginylation in mammals and yeast are under way, following the advances described in Abstract 158 (Kwon *et al.*, 2002). These studies include:

(i) Dissection of the substrate specificity of the *ATE1*-encoded R-transferases of *S. cerevisiae* and the mouse, including analyses of the mechanism of selective oxidation of N-terminal Cys (see Abstract 158) (*Rong-Gui Hu*). One of our aims here is to understand the physiological significance of differences in the activity of R-transferases toward N-terminal Cys versus its oxidized derivatives such as a cysteic acid residue. These projects include the development of new methods, based on cell-penetrating short peptides, to assess the *in vivo* state of

a reporter's N-terminal residue.

(ii) Construction and functional analyses of mouse strains (and cells derived from them) in which the expression of the *ATE1*-encoded Arg-tRNA-protein transferases (R-transferases) is selectively and conditionally abolished (or induced) in specific cell lineages during embryogenesis, or postnatally (*Christopher Brower*). This major set of projects will allow, among other things, a genetic dissection of N-terminal arginylation in specific cell types of adult mammal. (A nonconditional *ATE1-/-* genotype is embryonic lethal (Abstract 158)).

(iii) Analysis of chromosome stability and regulation of apoptosis in mouse *ATE1(-/-)* cells (*Jianmin Zhou and Christopher Brower*). This set of projects stems from the discovery of the function of the *S. cerevisiae* N-end rule pathway in the maintenance of chromosome stability (Rao *et al.*, 2001; Abstract 167), and from the conjecture (to be verified in this set of projects) that an analogous function in mammalian cells involves the arginylation (*ATE1*-dependent) branch of the N-end rule pathway.

(iv) Identification of *ATE1*-dependent circuits (i.e., the circuits that involve N-terminal arginylation) through the identification of mouse genes whose expression is significantly altered during embryonic development in *ATE1(-/-)* embryos, using microarray techniques, differential display and analogous methods with mRNA preparations from *ATE1-/-* and congenic +/+ embryos (*Jun Sheng*).

(v) Discovery of physiological substrates of R-transferases through the testing of putative substrates of caspases and calpains that bear secondary or tertiary destabilizing N-terminal residues (*Jianmin Zhou and Christopher Brower*). Another set of projects in this area involves systematic comparison, through immunoblotting, of the relative levels of a large number of different regulatory proteins (using, in particular, commercial antibodies to these proteins) in extracts from *ATE1(-/-)* cells versus their congenic +/+ counterparts (*Jun Sheng and Jianmin Zhou*). Positive hits in these quasirandom screens would identify either substrates of R-transferases or components of circuits that involve N-terminal arginylation.

References

Kwon, Y.T., Kashina, A.S., Davydov, I.V., Hu, R.-G., An, J.Y., Seo, J.W., Du, F. and Varshavsky, A. (2002) *Science* **297**:96-99.

Rao, H., Uhlmann, F., Nasmyth, K. and Varshavsky, A. (2001) *Nature* **410**:955-959.

160. Construction and analysis of mouse strains lacking the ubiquitin ligase UBR1 (E3α) of the N-end rule pathway

Yong Tae Kwon, Zanxian Xia, Ilia Davydov[1], Alexander Varshavsky

In mammals, UBR1/E3α is a major recognition component of the N-end rule pathway. We previously cloned and characterized the mouse *UBR1* gene and cDNA (Kwon *et al.*, 1998). Mouse UBR1 was expressed in *ubr1*Δ *S. cerevisiae*, and was found to rescue the activity of the N-end rule pathway in yeast, provided that mouse mHR6A or mHR6B (E2-14K) Ub-conjugating (E2) enzyme was coexpressed as well. (The endogenous yeast RAD6 E2 did not support the complementing activity of mouse UBR1 in *ubr1*Δ *S. cerevisiae*.) To address the function of *UBR1*, we constructed *UBR1(-/-)* mouse strains, using targeted mutagenesis in ES cells (Kwon *et al.*, 2001). The resulting deletion/disruption allele of *UBR1* lacked the segment encoding both type 1 and type 2 substrate-binding sites of UBR1. *UBR1(-/-)* mice lacked the UBR1 protein, were slightly smaller that congenic +/+ mice, but did not exhibit significant impairments in motor coordination. The expression of *UBR2* and *UBR3* mRNAs was slightly increased in the skeletal muscle of ad libitum-fed *UBR1(-/-)* mice, in comparison to congenic +/+ mice. In fasting mice, the expression of *UBR1, UBR2*, and *UBR3* mRNAs was significantly increased at least in the skeletal muscle. The magnitude of this increase, for *UBR2* and *UBR3* mRNAs, was larger in the skeletal muscle of fasting *UBR1(-/-)* mice, suggesting regulatory interactions among *UBR1*, *UBR2* and *UBR3*. Although *UBR1(-/-)* embryonic fibroblast (EF) cells lacked UBR1, the activity of the N-end rule pathway was found to be essentially unchanged in these cells, in comparison to +/+ EFs. Thus, there must exist at least one other functional counterpart of *UBR1* that is expressed at least in EF cells. Recent work has shown it to be *UBR2* (Abstract 161). On the other hand, experiments with muscle extracts from *UBR1(-/-)* mice indicated the absence of the N-end rule pathway from these extracts, in contrast to otherwise identical extracts from congenic +/+ mice. The absence of *UBR1* resulted in growth retardation throughout embryonic development; the 10-20% difference in mass between newborn *UBR1(-/-)* and +/+ pups was retained in adult mice as well. In *UBR1(-/-)* mice, the expression of fatty acid synthase mRNA was reduced, consistent with their (disproportionately) lower fat content. In addition, the expression of fatty acid synthase was not shut down upon fasting in *UBR1(-/-)* mice, in contrast to +/+ mice. We also analyzed plasma chemistry in +/+ and *UBR1(-/-)* mice, and found significant hypoglycemia in the latter, under both normal and starvation conditions (Kwon *et al.*, 2001). Our data strongly suggest that *UBR1(-/-)* mice are mosaics in regard to the activity of the N-end rule pathway, owing to differential expression of E3(s) that can substitute for the ubiquitin ligase UBR1 (E3α).

[1]*Present address: IGEN International, Inc., Gaithersburg, MD 20877.*

References

Kwon, Y.T., Reiss, Y., Fried, V.A., Hershko, A., Yoon, J.K., Gonda, D.K., Sangan, K., Copeland, N.C., Jenkins, N.A., Varshavsky, A. (1998) *Proc. Natl. Acad. Sci. USA* **95**:7898-7903.

Kwon, Y.T., Xia, Z.-X., Davydov, I.V., Lecker, S.H. and Varshavsky, A. (2001) *Mol. Cell. Biol.* **21**:8007-8021.

161. Apoptosis of spermatocytes and female lethality in mice lacking the UBR2 ubiquitin ligase of the N-end rule pathway

Yong Tae Kwon, Zanxian Xia, Ilia Davydov[1], Jee Young An, Jai Wha Seo, Alexander Varshavsky

The absence of gross defects in *UBR1(-/-)* mice suggested that the E3 function of the mouse N-end rule pathway is mediated by at least two functionally overlapping genes, one of which is *UBR1.* The previously identified mouse gene, termed *UBR2*, encodes a 200 kD protein highly similar to UBR1 (Kwon *et al.,* 1998). *UBR2(-/-)* mouse strains were recently constructed and characterized, revealing a sex-specific phenotype: male infertility and female lethality. The relative frequency of *UBR2(-/-)* males produced from heterozygous (+/- × +/-) matings was similar to that of +/+ males. Adult *UBR2(-/-)* males were outwardly normal, as judged by body weight and general behavior, but exhibited severe defects in spermatogenesis and were sterile. Until four weeks after birth, the size of their testes was close to that of congenic +/+ mice. However, during the 5th and 6th weeks, the mass of *UBR2(-/-)* testes decreased by ~2-fold. The degeneration of testes in *UBR2(-/-)* mice was caused by massive apoptosis of spermatocytes and their progeny, round spermatids. The number of apoptotic germ line cells in the mutant testes dramatically increased 5 weeks after birth. The first detectable abnormality of germ cell differentiation in the *UBR2(-/-)*testes was a much lower number of early meiotic spermatocytes at week 2. At week 3, when meiotic divisions of spermatocytes produce round spermatids, ~2,800 round spermatids per 100 seminiferous tubules were observed in the +/+ testes, but virtually no round spermatids could be detected in the mutant testes. This and related data suggested that defective meiosis in *UBR2(-/-)* male mice was the primary cause of their sterility. Despite the meiotic defect, a small number of cells that appeared to be round spermatids could be detected in the *UBR2(-/-)* testes after week 4. However, most of these cells did not continue to differentiate, and died through apoptosis, together with meiotic spermatocytes, after week 6. As a result, the testes of 2 months old *UBR2(-/-)*males were severely shrunk and vacuolized; they nearly completely lacked spermatids and spermatozoa. In contrast, the spermatogonia, Sertoli cells, and Leydig cells remained apparently intact in the *UBR2(-/-)* testes. Thus, the absence of UBR2 (but not of its close homolog UBR1; Abstract 160) leads to a severe defect in spermatogenesis that stems primarily, if not exclusively, from a meiotic defect. Further analysis of this phenotype showed that *UBR2(-/-)* spermatocytes are arrested largely at the pachytene stage of meiosis, and fail to form the synaptonemal complex that holds together the homologous chromosomes and is essential for meiotic recombination. Recent GST-pulldown binding assays with purified N-end rule substrates and extracts from *S. cerevisiae* overexpressing mouse UBR2 have shown that UBR2 can recognize N-degrons *in vitro*. Thus, mouse UBR2 as an E3 component of the N-end rule pathway that functionally overlaps with the previously known E3 of this pathway, UBR1 (E3α).

Yet another feature of the *UBR2 (-/-)* phenotype was extremely low frequency of the *UBR2(-/-)* female progeny from heterozygous (+/- × +/-) matings, in contrast to the normal (mendelian) frequency of *UBR2(-/-)* males. Strikingly, the rare *UBR2(-/-)* females that were born were fertile and apparently normal otherwise. This phenotype is being analyzed. It is likely to be caused by perturbed X-chromosome inactivation in early *UBR2(-/-)* female embryos.

To investigate the functional interaction of UBR1 and its close homolog UBR2, we used *[UBR1(+/-) UBR2(+/-)]* mice to produce *[UBR1(-/-)UBR2(-/-)]* mouse strains. Disruption of both *UBR1* and *UBR2* was lethal: none of the *[UBR1(-/-)UBR2(-/-)]* embryos survived beyond day E12 (Y. T. Kwon and J. W. Seo, unpublished data). Whereas the double-mutant male embryos died at ~E9.5, most of their female counterparts died significantly earlier. Remarkably, *UBR1(-/-)UBR2(-/-)* embryonic fibroblasts did contain the N-end rule pathway, albeit of significantly lower activity, indicating the presence of at least one other (third) E3 component of this pathway

[1]*Present address: IGEN International, Inc., Gaithersburg, MD 20877*

Reference

Kwon, Y.T., Reiss, Y., Fried, V.A., Hershko, A., Yoon, J.K., Gonda, D.K., Sangan, K., Copeland, N.C., Jenkins, N. A., Varshavsky, A. (1998) *Proc. Natl. Acad. Sci. USA* **95**:7898-7903.

162. Construction and analysis of mouse strains lacking the UBR3 ubiquitin ligase

Takafumi Tasaki, Yong Tae Kwon, Alexander Varshavsky

Kwon *et al*. (1998) identified two distinct mouse (and human) genes, termed *UBR2* and *UBR3*, which encode proteins that are similar to mouse UBR1 (E3α), the previously characterized E3 of the N-end rule pathway (Abstracts 159 and 160). In contrast to the highly similar mouse UBR1 and UBR2 proteins (47% identity and 68% similarity), the mouse UBR3 protein, while clearly a member of the UBR family is less similar to UBR1 (25% identity and 51% similarity) and UBR2 (25% identity and 48% similarity). In addition, mouse UBR3 lacks some of the residues in its N-terminal region that have been shown to be essential for the function of yeast UBR1, and are also present in mouse (and human) UBR1 and UBR2. We mapped and partially sequenced the mouse *UBR3* gene, and more recently constructed *UBR3(-/-)* mouse strains. *UBR3(-/-)* mice are viable, and are being characterized.

Reference

Kwon, Y.T., Reiss, Y., Fried, V.A., Hershko, A., Yoon, J.K., Gonda, D.K., Sangan, K., Copeland, N.C., Jenkins, N.A., Varshavsky, A. (1998) *Proc. Natl. Acad. Sci. USA* **95**:7898-7903.

163. Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain

Fangyong Du, Federico Navarro-Garcia[1], Zanxian Xia, Takafumi Tasaki, Alexander Varshavsky

Two substrate-binding sites of UBR1, the E3 of the N-end rule pathway in *S. cerevisiae*, recognize basic (type 1) and bulky hydrophobic (type 2) N-terminal residues of proteins or short peptides. A third substrate-binding site of UBR1 targets CUP9, a transcriptional repressor of the peptide transporter PTR2, through an internal (non-N-terminal) degron of CUP9 (Byrd *et al.*, 1998; Turner *et al.*, 2000). Previous work (Turner *et al.*, 2000) demonstrated that dipeptides with destabilizing N-terminal residues allosterically activate UBR1, leading to accelerated *in vivo* degradation of CUP9, a strong decrease of CUP9 concentration, and the induction of PTR2 expression. Through this positive feedback, *S. cerevisiae* can sense the presence of extracellular peptides, and react by accelerating their uptake. In the present work (Du *et al.*, 2002), we found that dipeptides with destabilizing N-terminal residues cause dissociation of the C-terminal autoinhibitory domain of UBR1 from its N-terminal region that contains all three substrate-binding sites. This dissociation, which allows the interaction between UBR1 and CUP9, is strongly increased only if both type 1 and type 2 binding sites of UBR1 are occupied by dipeptides. An aspect of autoinhibition characteristic of yeast UBR1 was also observed with mammalian (mouse) UBR1 (E3α). Autoinhibition has not been reported previously for Ub ligases. UBR1, UBR2 and UBR3, the mouse homologs of *S. cerevisiae* UBR1, contain the conserved (and similarly arranged) UBHC, RING-H2, and UBLC domains similar to those of yeast UBR1. In addition, mouse UBR1 and UBR2 contain binding sites for the type 1 and type 2 destabilizing N-terminal residues. Moreover, the functional properties of these sites, such as the ability of type 1 dipeptides to stimulate the degradation of type 2 N-end rule substrates, are also similar to those of yeast UBR1. Finally, an aspect of autoinhibition characteristic of yeast UBR1 was also observed with mouse UBR1. Thus, it is highly likely that metazoan E3s of the UBR family are also controlled through autoinhibition, similarly to yeast UBR1. The *S. cerevisiae* UBR2 E3 and mouse UBR3 E3, whose functions remain unknown, are not a part of the N-end rule pathways in the respective organisms (H. Rao, T. Tasaki, Y.T. Kwon, and A. Varshavsky, unpublished data). Thus, the (postulated) autoinhibition of yeast UBR2 and mouse UBR3 may be regulated by naturally occurring small compounds distinct from dipeptides. Identifying these compounds will provide a clue to still unknown physiological roles of the UBR-family Ub ligases that function outside the N-end rule pathway. The discovery of autoinhibition in the Ub ligases of the UBR family (Du *et al*., 2002) indicates that this regulatory mechanism may control the activity of other Ub ligases as well. Our findings also suggest that natural compounds (either small molecules or proteins) may regulate the activity of diverse Ub-dependent pathways through the suppression or induction of autoinhibition in Ub ligases.

[1]*Present address: University of Madrid, Spain.*

References

Byrd, C., Turner, G. and Varshavsky, A. (1998) *EMBO J.* **17**:269-277.

Du, F., Navarro-Garcia, F. Xia, Z., Tasaki, T. and Varshavsky, A. (2002) *Proc. Natl. Acad. Sci. USA*. In press.

Turner, G.C., Du, F. and Varshavsky, A. (2000) *Nature* **405**:579-583.

164. Quantitative analyses of interactions between components of the N-end rule pathway and their substrates or effectors

Zanxian Xia, Alexander Varshavsky

Detailed understanding of the N-end rule pathway requires, among other things, the knowledge of equilibrium binding constants (in some cases, of the corresponding rate constants as well) for the reversible interactions between UBR1 and its effectors or substrates. We are carrying out these measurements using the fluorescence polarization (FP) technique, with purified *S. cerevisiae* UBR1 (or its fragments) and a variety of UBR1 ligands, including peptides with destabilizing N-terminal residues. These analyses will be expanded to include other physiological ligands of UBR1 such as the RAD6 Ub-conjugating enzyme, and also CUP9, a homeodomain repressor (Abstract 163) recognized through its C-terminal degron by a distinct (third) substrate-binding site of UBR1.

165. The UFD4 ubiquitin ligase lacking the proteasome-binding region catalyzes ubiquitylation but is impaired in proteolysis

Youming Xie, Alexander Varshavsky

We have previously shown that *S. cerevisiae* UBR1 and UFD4, the E3 enzymes of, respectively, the N-end rule pathway and the UFD (Ub/fusion/degradation) pathway, directly interact with specific subunits of the 26S proteasome (Xie and Varshavsky, 2000). These and other recent results suggested a route for the substrate's delivery to the proteasome distinct from the previously explored proteasome binding to a substrate-linked multi-Ub chain. The GST (glutathione S-transferase) pulldown assay was used to test nine subunits of the 19S particle for binding to UFD4, identifying RPT6 as the UFD4-binding subunit in that set (Xie and Varshavsky, 2000). More recently, we advanced this analysis for the UFD4 Ub ligase, and showed that it interacts with RPT4 and RPT6, two of the ~17 subunits of the 19S particle (Xie and Varshavsky, 2002). The 201-residue N-terminal region of UFD4 was found to be essential for its binding to RPT4 and RPT6. UFD4$^{\Delta N}$, which lacks this N-terminal region, is apparently wild-type in ubiquitylating UFD substrates *in vivo*, but is impaired in conferring short half-lives on these substrates. We suggest that interaction of a targeted substrate with the 26S proteasome involves contacts of specific proteasomal subunits with the E3 of an E3-substrate complex, with the substrate's multi-Ub chain, and with the substrate itself. This broadly spaced, multisite binding may serve to slow down substrate's dissociation from the proteasome and to

facilitate the unfolding of substrate upon ATP-dependent movements of the chaperone subunits of the 19S particle (Xie and Varshavsky, 2002).

References

Xie, Y. and Varshavsky, A. (2000) *Proc. Natl. Acad. Sci. USA* **97**:2497-2502.

Xie, Y. and Varshavsky, A. (2002) Submitted for publication.

166. Characterization of the yeast ubiquitin ligase UBR2

Hai Rao, Youming Xie, Yong Tae Kwon, Alexander Varshavsky

The 190 kD UBR2 is the only *S. cerevisiae* protein that is highly similar in sequence to the 225 kD UBR1, the E3 of the N-end rule pathway. We found, using two-hybrid assays, that the UBR2 interacts with the Ub-conjugating enzyme RAD6, the E2 enzyme of the N-end rule pathway. In contrast to UBR1, UBR2 does not recognize N-end rule substrates. Moreover, overexpression of UBR2 partially inhibits the N-end rule pathway, presumably because of competition for the RAD6 E2 enzyme, a ligand in common between UBR1 and UBR2. Conversely, the N-end rule pathway is more active in a *ubr2*Δ strain. Phenotypes of mutants overexpressing or lacking UBR2 suggest a role for UBR2 in sporulation. Physiological substrates and degrons recognized by this UBR1-like E3 enzyme remain to be identified.

167. Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability

Hai Rao, Frank Uhlmann[1], Kim Nasmyth[2], Alexander Varshavsky

The sister chromatids of a replicated chromosome are pulled apart by the centromere-attached microtubules that emanate from spindle poles at opposite sides of the cell. However, until the onset of anaphase the forces pulling sister chromatids apart are counteracted by cohesion that holds sisters together. A multisubunit complex termed cohesin is essential for sister chromatid cohesion in both fungi and metazoans. In the budding yeast *S. cerevisiae*, cohesin contains four subunits, termed SMC1, SMC3, SCC1 (MCD1, RAD21), and SCC3. Perturbations of chromosome segregation are among the causes of human cancer and other diseases. Previous work by the laboratory of K. Nasmyth demonstrated that at the metaphase-anaphase transition in *S. cerevisiae*, the *ESP1*-encoded protease called separase, cleaves SCC1, a 63 kD subunit of cohesin. The resulting 33 kD C-terminal fragment of SCC1 bears N-terminal arginine, a destabilizing residue in the N-end rule. We have found that the SCC1 fragment is short-lived *in vivo* ($t_{1/2}$ ~2 min), being degraded by the N-end rule pathway. Overexpression of a long-lived derivative of the SCC1 fragment is lethal. These results led us to examine chromosome stability in *ubr1*Δ cells (which lack the N-end rule pathway), revealing a greatly increased frequency of chromosome loss in these cells (Rao *et al.,* 2001). Further, we demonstrated that the bulk of increased chromosome loss in ubr1 cells is caused by metabolic stabilization of the ESP1-produced SCC1 fragment. This fragment is the first physiological substrate of the N-end rule pathway that is targeted through a substrate's N-terminal residue. A number of yeast proteins bear putative cleavage sites for the ESP1 separase, suggesting other physiological N-end rule substrates and other functions of the N-end rule pathway (Rao *et al.,* 2001).

[1]*Imperial Cancer Research Fund, London WC2A 3PX, UK*
[2]*Institute of Molecular Pathology, Vienna, Austria*

Reference

Rao, H., Uhlmann, F., Nasmyth, K. and Varshavsky, A. (2001) *Nature* **410**:955-959.

168. Dissection of c-MOS degron

Jun Sheng, Akiko Kumagai, William G. Dunphy, Alexander Varshavsky

c-MOS (called MOS below) was originally identified as a cellular homolog of the v-MOS oncoprotein of Moloney murine sarcoma virus. MOS is a serine/threonine protein kinase that is normally expressed largely, if not exclusively, in oocytes and spermatocytes. MOS functions as a regulator of oocyte maturation. A fully-grown (stage-VI) *Xenopus* laevis oocyte is arrested in the G2 phase of the first meiotic cell cycle. Oocyte maturation, initiated by progesterone, involves the completion of meiosis I, that is followed by meiosis II and the arrest at metaphase of meiosis II. At this stage, the mature oocyte, called an egg, is ready for fertilization. MOS phosphorylates, and thereby activates, the kinase MEK1. In the MAP cascade's terminology, MOS is a MAP kinase kinase kinase. The synthesis of MOS begins shortly after progesterone stimulation, and ceases near the end of oocyte maturation. The concentration of MOS is controlled both through its synthesis and through its conditional degradation by the Ub system.

Studies by Sagata and colleagues (Nishizawa *et al.*, 1992, 1993) have shown that wild-type MOS, bearing the N-terminal Pro residue, is short-lived upon its expression in oocytes, whereas the otherwise identical MOS derivative bearing N-terminal Gly is long-lived. These and related findings led Nishizawa *et al.* (1992, 1993) to propose the "second-codon rule," in which the N-terminal Pro residue of MOS targets MOS for degradation. In the N-end rule terminology, the above conjecture meant that the MOS protein contains an N-degron whose N-terminal Pro is recognized by a specific Ub ligase. One difficulty with this model was that N-terminal Pro, in the context of previously characterized N-end rule reporters, was clearly a stabilizing residue.

To address this problem, we analyzed the MOS degron in greater detail, found it to be a portable degron, and demonstrated that, contrary to the model above, the N-terminal Pro residue of c-MOS is entirely dispensable for its degradation if Ser-2 (encoded Ser-3) of c-MOS is replaced by a small nonphosphorylatable residue such as Gly (Sheng *et al.*, 2002). The dependence of c-MOS degradation on N-terminal Pro was shown to be caused by a Pro-mediated down-regulation of the net phosphorylation of Ser-2, a modification that halts c-MOS degradation in oocytes. Thus, the N-terminal Pro residue of c-MOS is not

a recognition determinant for a ubiquitin ligase, in agreement with earlier evidence that Pro is a stabilizing residue in the N-end rule.

References

Nishizawa, N. *et al.* (1992) *EMBO J.* **11**:2433-2446.
Nishizawa, N. *et al.* (1993) *EMBO J.* **12**:4021-4027.
Sheng, J., Kumagai, A., Dunphy, W. and Varshavsky, A. (2002). *EMBO J.*. In press.

Publications

Du, F., Navarro-Garcia, F. and Varshavsky, A. (2002) Pairs of dipeptides synergistically activate the binding of substrate by ubiquitin ligase through dissociation of its autoinhibitory domain. *Proc. Natl. Acad. Sci. USA*. In press.

Kwon, Y.T., Xia, Z.-X. Xia, Davydov, I.V., Lecker, S.H. and Varshavsky, A. (2001) Construction and analysis of mouse strains lacking the ubiquitin ligase UBR1 (E3α) of the N-end rule pathway. *Mol. Cell. Biol* **21**:8007-8021.

Kwon, Y.T., Kashina, A.S., Davydov, I.V., Hu, R.-G., An, J.Y., Seo, J.W., Du, F. and Varshavsky, A. (2002) An essential role of N-terminal arginylation in cardiovascular development. *Science* **297**:96-99.

Kwon, Y.T., Xia, Z.-X., Davydov, I.V., An, J.Y., Seo, J.W. and Varshavsky, A. (2002) Apoptosis of spermatocytes and female lethality in mice lacking the UBR2 ubiquitin ligase of the N-end rule pathway. Manuscript in preparation.

Rao, H., Uhlmann, F., Nasmyth, K. and Varshavsky, A. (2001) Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability. *Nature* **410**:955-959.

Sheng, J., Kumagai, A., Dunphy, W. and Varshavsky, A. (2002) Dissection of c-MOS degron. *EMBO J.* In press.

Varshavsky, A. (2002) Proteolysis. *Encyclopedia of Genetics* (Academic Press, NY), v. **3**, pp. 1573-1575.

Varshavsky, A. (2002) Ubiquitin. *Encyclopedia of Genetics* (Academic Press, NY), v. **4**, pp. 2091-2093.

Varshavsky, A. (2002) Recent studies of the ubiquitin system and the N-end rule pathway. *Harvey Lectures.* In press.

Xie, Y. and Varshavsky, A. (2001) RPN4 is a ligand, substrate, and transcriptional regulator of the 26S proteasome: A negative feedback circuit. *Proc. Natl. Acad. Sci. USA* **98**:3056-3061.

Xie, Y. and Varshavsky, A. (2002) The UFD4 ubiquitin ligase lacking the proteasome-binding region catalyzes ubiquitylation but is impaired in proteolysis. Submitted for publication.

Professor: Barbara J. Wold
Senior Research Fellow: Jeong Kyo Yoon
JPL Visiting Associate: Eric Mjolsness
Member Professional Staff: Hiroke Shizuya
Visitor: Liberia Berghella
Postdoctoral Scholar: Henrik Jonsson, Brian Williams
Graduate Students: Tristan DeBuysscher, Christopher Hart, Tony Kirilusha, Tracy Teal
Research and Laboratory Staff: Jennifer Ambroggio, Sagar Damle, Leslie Dunipace, Richel Gwirtz, Parvin Hartsteen, Brandon King, Dee Page, Diane Trout, Shuling Wang, Yan Wu

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Summary: In the Wold group we are interested in the composition, evolution and function of regulatory networks that govern how mammalian cell fates are specified and executed during development and during regeneration. This theme extends to related lineages of adult stem cells for our model system and to the way in which cells of this same lineage become tumorgenic. Approaches we are taking to these problems increasingly use genome-wide and proteome-wide assays. To do this some of our efforts now include development of new wet-bench genomic technology and computational methods, the latter developed in an on-going partnership with Professor Eric Mjolsness of JPL/University of California Irvine.

A key challenge is to understand the regulatory events that drive the progression from multipotential precursor cells to determined unipotential progenitors and then to fully differentiated cells. We are currently studying these cell states and transitions using microarray gene expression analysis, global protein:DNA interaction measures, mass spec based proteomics of multiprotein complexes, and comparative genomics. The mouse is our primary experimental animal, and the focal developmental lineage arises from paraxial mesoderm to produce muscle (also bone, skin and fat, among other derivatives). Skeletal myogenesis is governed by both positive- and negative-acting regulatory factors. The MyoD family of four closely related, positive-acting transcription factors are key. Upon transfection each can drive nonmuscle recipient cells into the myogenic pathway. Given their extraordinary power to drive or redirect a cell fate decision, a central goal is to understand how the regulatory network in which they are embedded directs cell fate selection and execution of the differentiation transition. At cellular and molecular levels, it is clear that negative regulators of skeletal myogenesis are probably just as important for regulating the outcome as are the positive regulators. The interaction between positive and negative acting regulators continues to be of particular interest. Multiple negative regulators of skeletal muscle are expressed in multipotential mesodermal precursors and in proliferating muscle precursors (myoblasts). It is generally believed that some of these are important for specifying and/or maintaining precursor cells in an undifferentiated state, though exactly how the system works is unknown.

To define this myogenic regulatory network more comprehensively, we have developed a major collaborative effort with the Deshaies lab here and the John Yates lab at Scripps to modify and apply MudPIT mass spectrometry, coupled with dual affinity epitope tagging, to characterize multiprotein complexes. To define the *cis*-acting regulatory elements to which these protein complexes bind we have entered into a collaboration with Eric Greene at NIH to isolate and sequence genes from our network from ten vertebrate species each. The computational tools described below are now being used to find candidate conserved regulatory elements, and these are, in turn, being subjected to rapid functional assays via lentiviral mediated transgenesis. These same tools are being used to analyze data from multiple species of worms related, in differing degrees, to *C. elegans*. This project is in partnership with the Sternberg lab and Hiroke Shizuya here at Caltech, and the DOE Joint Genomics Institute, where large scale DNA sequencing is done. In this project large insert, random shear libraries were made for two new worm species, and genes from several regulatory networks, including the myogenic one are being isolated and sequenced for comparative analysis. In addition to clarifying how many and which worm genomes give us the most leverage for identifying functionally important noncoding elements in the genome, we hope to gain insights into the evolution of myogenic networks across large phylogenetic distances between vertebrates, worms and flies.

Our collaboration with Dr. Timothy Triche and colleagues at Children's Hospital is giving us a picture of how the myogenic developmental pathway relates to cells of the myogenic lineage when they run amok in cancer. Transcriptome analysis of over 100 rhabdomyosarcomas has given us several new insights into the nature of these tumors, plus identification of previously unappreciated signaling pathways that are candidates for causal contributions to tumor properties. Of particular interest was a surprising re-classification of one subgroup of tumors. These are, by histological criteria, of the alveolar class. However, by expression profiling and subsequent computational analysis, these tumors proved to be very different from classic Alveolars and more similar to the other major class (Embyonal). Retrospectively, we were able to relate this to the absence of a chromosomal translocation that characterizes the most alveolar tumors. This analysis also showed, surprisingly, that the genes whose expression best separates conventional alveolar and embryonal types does not correlate with their histological

appearance, but rather refers to other molecular differences. We now postulate that these "invisible" differences may have more powerful prognostic capacity than conventional histopathology, since the two tumor classes differ significantly in outcome, than does histopathological classification. This has implications for treatment pathways and prognosis.

Muscle satellite cells are muscle stem cells of the adult, and they are responsible for all muscle regeneration following injury or degenerative insult. We want to understand the lineal origin, cell cycle regulation, and regulation of myogenic status for this self-renewing progenitor cell population. D. Cornelison initiated this work as part of her PhD thesis in the lab by studying in satellite cells the expression and function of a panel of 80 different developmental regulatory genes, growth and trophic factor receptors, cdk/cyclin inhibitory proteins, and cdk/cyclin complexes in the context of mouse muscle regeneration for wild-type and MRF mutant animals. Marie Csete went on to study these stem cells and others from diverse tissues with respect to their responses to major environmental cues, focusing on oxygen availability that is often disrupted by injury. Collaboration with David Anderson's lab on campus and Ron McKay's at the NIH led to the conclusion that oxygen levels normally used in cell culture are both a physiologically high and deleterious. Lower, more physiological levels give dramatic improvement in yields of dopaminergic neurons in both CNS and PNS stem cell cultures. Oxygen levels were also found to influence the selection of cell fate in culture, leading to the provocative possibility that the oxygen microenvironment for a progenitor cell during development *in vivo* might affect cell fate selection in the brain. There are also intriguing effects of oxygen levels on muscle and fat developmental pathways where aphysiologically high levels of oxygen favor the fat pathway and lower normoxic levels favor accelerated muscle differentiation. The current challenge is to identify the metabolic and regulatory pathways that are responsible for these oxygen effects.

A different bHLH class regulator was discovered by Jeong Yoon in the lab, and it ultimately has made somite formation and vertebrate body segmentation a topic of investigation in the lab. The regulator is called p-Mesogenin1 because it functions early in the specification and subdivision of unsegmented paraxial mesoderm. By analogy with other bHLH regulators, it is likely to function as a sequence specific DNA binding protein. Jeong Yoon in the lab previously showed by gain of function analysis in frog embryos and loss of function studies in a mouse p-Meso knockout that this protein is essential for vertebrate body segmentation (somite formation) and for subsequent cellular survival and maturation of all trunk and tail paraxial mesoderm. Recent work has preliminarily identified regulatory elements responsible for its novel pattern of expression, and it is slated for upcoming multi-genome sequencing and comparative analysis.

An entirely new project for the lab is Tracy Teal's. She is a joint graduate student with Dianne Newman in the Division of Geology and Planetary Science. The topic is environmental bacterial biofilms, and the goal is identify, visualize, and ultimately understand the multiple different metabolic cell states that comprise a biofilm at different stages of its development and under differing environmental stimuli. The degree to which principles and regulatory strategies used by metazoans during development are or are not employed by bacteria in creating biofilm structures is being probed by marking bacteria with multiple GFP derivatives driven by genes that are believed to be differentially expressed within biofilms.

169. Lowered oxygen culture affects skeletal muscle satellite cell yield, development and phenotype

Marie Csete, Jean Walikonis, Shuling Wang***

Satellite cells are normally quiescent cells present in small numbers under skeletal muscle basal lamina. Satellites re-enter the cell cycle when skeletal muscle is traumatized, and produce myoblasts and ultimately new muscle fibers. They are thought to be the major source of new skeletal muscle after trauma or degeneration. An adult muscle fiber culture model of regeneration was established in the lab by D. Cornelison (1). That work demonstrated that satellites in culture activate in a relatively synchronous fashion, with single satellites emerging from the basal lamina at about one day in culture and the first satellite division common at 48 hr, with new muscle fiber apparent at 96 hr. A characteristic expression pattern of muscle transcription factors (MyoD, myf5, myogenin, and MRF4) accompanies these morphologic patterns.

Traditional tissue culture is usually conducted at oxygen levels that are aphysiologically high, even for skeletal muscle, a generally "high"-oxygen tissue. On average skeletal muscle tissue oxygen levels are approximately 6% O_2. Csete and Walikonis compared early satellite regeneration under physiologic oxygen levels vs. usual 20% O_2 culture conditions. Using sequential 12-hr BrdU pulses to label satellites adherent to the fibers, we found twice as many satellites proliferating at each time point (up to 60 hr) in physiologic oxygen vs. 20% O_2. Immunohistochemical labeling of satellites using immunostaining for the presence of c-Met receptors (an operational molecular marker of quiescent and recently activated muscle stem cells) confirmed the presence of twice as many satellites on fibers in low oxygen conditions at 24 and 48 hrs. In parallel studies using human myoblast cultures, we confirmed that these cells undergo enhanced proliferation under 2% O_2 conditions compared to traditional 20% O_2 cultures.

Single-cell RT-PCR was also used to assess the satellite developmental process. Individual satellite cells were assayed for the expression of the four major muscle regulatory transcription factors of the MRF family: myf5, MyoD, myogenin and MRF4. Myogenin and MyoD expression is more prominent at 24 and 48 hr under low oxygen conditions than under 20% O_2. At 24 hr, twice as many satellites in low oxygen have simultaneous

expression of three MRFs. Both findings suggest that the satellites are further along in their developmental pathway under physiologic oxygen conditions. This work, together prior work with neural stem cells, raises questions about which pathways are responding to oxygen variation, and large-scale expression surveys are one obvious pathway to explore and narrow the possibilities.

*University of Michigan, School of Medicine
**University of California, Irvine

Reference

(1) Cornelison, D.D.W. and Wold, B.J. (1997) *Dev. Biol.* **191**:270-283.

170. Characterization of pediatric sarcomas by microarray-based gene-expression studies

Sagar Damle, Joe Roden, Ben Bornstei, Dennis DeCoste*, Eric Mjolsness***

The central model system for our lab's developmental biological studies is paraxial mesoderm with emphasis on the myogenic pathway. In cancer biology, tumors derived from these cell lineages are the sarcomas of childhood, and those in the muscle lineage are called rhabdomyosarcomas. This work begins by probing a well-defined and large set of rhabdomyosarcomas via large scale expression analysis and ask a series of questions including: How does gene expression in the tumors relate to that seen in normal development? Can we detect groups of genes whose patterns of expression predict how aggressive a tumor will be? Can we identify patterns of genes that classify tumors into subgroups that correspond to those currently generated by pathologists? Is there a group of genes whose expression that corresponds with presence of two known chromosomal translocation events that are believed to be causal for one subtype of tumor? Is there a set of genes that can help explain the bizarre observation that a small subset of these tumors are highly metastatic, yet essentially benign, while others that look histologically similar are rapidly fatal? We then seek to relate what is known about the identities of signature genes with signaling pathways, cell shape and migration, gene regulation etc., to identify candidate genes or pathways for future drug treatments.

Two histopathologically different kinds of rhabdomyosarcoma (RMS), alveolar and embryonal RMS are associated with distinct clinical characteristics and different cytogenetic properties. Most alveolar class RMS are characterized by a t(2:13) or t(1:13) chromosomal translocation which results in the fusion of the DNA-binding motifs of either PAX7 or PAX3 and the carboxy-terminal activation domain of forkhead gene. Embryonal class RMS instead show allelic loss of regions of chromosome 11 thought to contain tumor suppressor genes. In a long-term collaboration with the Triche Laboratory and Children's Hospital LA, Affymetrix microarrays (U133A/B) were used to measure the gene-expression profiles of 31,728 unigene-derived features in 56 RMS tumors (35 ERMS 21, ARMS). Statistical analysis was performed using a 2-class T-test. Despite the relatively common muscle backgrounds of these two cell-types, we were able to identify several hundred differentially expressed genes (with a p-value less than 0.05). Members of this subset have been identified as having roles in specific cell-cycle regulatory and apopotosis-related pathways. Surprisingly, cannabinoid receptor 1, expressed normally in the hippocampus and responsible for phosphorylation of focal adhesion kinase (FAK), is overexpressed in ARMS elative to EMS. FAK, in turn, is upstream of several signaling pathways including adhesion-dependent survival and cell motility and inhibits apoptosis through pathways that inhibit degradation of AKT. FAK activation may explain the relative severity of prognosis and incidence of metastasis seen in ARMS. Alternatively, the transcription factor FoxF1 and its upstream regulator, Shh, were repressed in many ERMS, with corresponding upregulation of antagonist and growth factor, BMP4.

Other more sophisticated analyses, including both supervised and unsupervised clustering algorithms, have been applied in further characterizing the expression patterns seen in these tumors. Artificial neural networks (Bornstei; JPL), support vector machines (DeCoste; JPL) and other machine-learning algorithms are being used to identify genes whose expression differs across PAX3/PAX7 (ARMS), prognosis and metastasis boundaries in addition to the ARMS/ERMS divide. Gene subsets gathered from these lines of analysis have allowed for a finer parsing of tumor-types. A 2-dimensional plot of the PCA-transformed tissue-space in which the genespace was restricted to these subsets (Joe Roden; JPL) has helped to provisionally reclassify translocation negative ARMS tumors on the basis of their ERMS-like gene expression patterns. The possibility that these tumors also share the more favorable ERMS prognosis and the inevitable power this will allow in the customization of cancer treatment remains to be seen, but the prospects are encouraging.

*Machine Learning Systems Group, Jet Propulsion Laboratories
**JPL/Computer Science Department, University of California, Irvine

171. Gene expression and metabolic organization in *Shewanella oneidensis* biofilms

*T.K. Teal, B.J. Wold, D.K. Newman**

Bacteria have traditionally been viewed as living a primarily planktonic lifestyle. In the last decade however, it has become more apparent that bacteria spend much of their lives as surface-attached microbial communities, i.e., biofilms. These films have considerable 3-dimensional structure, meaning that the environment within different areas of the biofilm can vary markedly. These biofilms are prevalent in natural as well as man-made systems. Because of their medical and environmental importance, biofilms have recently become the subject of more intense study. We are interested in the process of biofilm formation and the metabolic organization of the biofilm. While more has become known about the biofilm lifestyle, including the existence

of intercellular signaling pathways, very little is understood about the temporal and spatial metabolic states within the biofilm and how they influence further biofilm development. This collaboration between Newman and Wold labs is particularly centered on probing similarities and differences in the strategies used in forming 3-D developing biofilms, comparing and contrasting the strategies used with those used in metazoan development. It is secondarily centered on implementing genomic scale assays, as called for in the project.

To investigate these topics we will be using biofilms comprised of Shewanella oneidensis strain MR-1. *S. oneidensis* is an environmentally important class of bacteria. It is a facultative anaerobe with remarkable respiratory versatility. Its genome has been sequenced, and it is relatively easy to manipulate genetically. We have started by investigating which parts of *S. oneidensis* biofilms are metabolically active. To do this we have tagged the ribosomal promoter rrnBPI with the unstable GFP gfp(AAV) and cloned it into a plasmid that is stable in MR1. Fluorescence levels from this promoter/GFP system have been shown to be a good indicator of cell growth and replication. We are growing the biofilms in a flow cell system and imaging them using 2-photon fluorescence microscopy. Using this technique we can correlate the level of fluorescence we see using microscopy with metabolic activity and can determine the regions of the biofilm in which cells are the most metabolically active as well as explore how quickly this state can change in response to environmental perturbations. Additional constructs using promoters sensitive to other metabolic parameters are in progress.

To gain a better understanding of the process of biofilm formation, we are interested in the genes being expressed as the biofilm develops. We have created a test DNA microarray with 75 selected genes from MR-1 thought to be involved in different aspects of biofilm formation. We will assay *S. oneidensis* biofilms for gene expression over time, and perhaps selected on the basis of GFP marker gene expression. We anticipate that we will see expression of genes important in biofilm attachment and growth as well as those involved in cell-cell signaling, or quorum sensing. Using this data we will then use 'classical genetics' techniques to determine the precise role of the identified genes. Ultimately we hope to develop a computational model of the key genetic networks involved in biofilm formation and cell-to-cell communication.

**Assistant Professor of Geobiology and Environmental Engineering Science, Caltech*

References

Bassler, B.L. (1999) *Curr. Opin. Microbiol.* **2**:582-587.

Hernandez M.E. and Newman D.K. (2001) *Cell Mol. Life Sci.* **58**:1562-1571.

Whitely M. *et al.* (2001) *Nature* **413**:860-864.

172. Analysis of transcriptional protein complexes via MudPIT mass spectrometry

Leslie Dunipace, Johannes Graumann, Jae Hong Seoul

It is difficult to overestimate the usefulness of learning the *in vivo* protein associations that mediate a cellular process. This project focuses on protein complexes involved in regulating gene expression and is part of a larger collaborative effort between our lab, the Deshaies lab, and John Yates' lab at Scripps. The overall collaboration has the initial goal of modifying and then applying at relatively high throughput, a mass spectrometry based method for mapping protein interactions *in vivo*. This approach couples MudPIT (MultiDimensional Protein Identification Technology) which originated in the Yates lab, with a dual affinity protein tagging / sample retrieval system constructed in the Deshaies group called HTM/HPM. Leslie has now used the tag to establish 25 yeast strains, each carrying the product of targeted recombination at a selected gene, so that the protein produced (from its normal regulatory sequences in the chromosome) carries an affinity tag at the carboxy-terminal end. This affinity tag contains two epitopes (His-9 and Myc-9) separated by a protease site. The His and Myc tags are used for sequential and rapid affinity purification. The purpose of the affinity steps is to enrich the sample for the tagged protein together with proteins associated with it, while eliminating unassociated proteins. Dual affinity is designed to eliminate the need for gel purification and "band-cutting," which has been a rate-limiting step in sample preparation in similar studies in the Deshaies lab and elsewhere in the past. The mass-spec analysis that follows identifies peptides in the sample by reference to the known genome and its predicted proteome, using a version of the Sequest software developed by Yates and colleagues. By increasing throughput, we aim to take entire groups of genes involved in a single cellular process or structure and gain a high quality mass spec map of the *in vivo* associations involved.

The 25 yeast strains were vetted for expression of the tagged protein by Western blotting against the Myc tag. Conditions for affinity retrieval were optimized and initial testing was performed by focusing on a relatively well-known, multiprotein complex (the chromatin remodeling complexes of which GCN5 is a component). Working in the Yates group, Johannes generated very encouraging and reproducible mass spec mapping results for tagged GCN5. All previously documented GCN-5 associated proteins were detected (except histone associations, which this cell preparation might not have been expected to retain), and the analysis also presented a few new reproducible candidate associations. Scale-up to the remaining 24 tagged strains is in progress, as is migration and installation of the instrumentation at Caltech. The longer-term biological goals are many in our groups, but a priority for the Wold group is on transcriptional complexes, and on modifying the process it for targeting mammalian regulatory complexes and pathways.

A modest transcriptionally oriented project in yeast will be to characterize the protein partners of all bHLH class regulators in yeast. The same-tagged factors will also be used in chromatin immunoprecipitation. For studies in mammalian systems, Leslie generated a family of retroviral vectors that can be used to make transgenic mice or to mediate expression in cultured mouse or human cells. Two such vectors – one placing the dual affinity HPM tag at the amino terminus of the target protein (gene) and the other at the carboxy terminus were constructed. The lead mammalian protein for our study, the myogenic transcription factor MyoD, was introduced into both and tested for bioactivity. The carboxy-tagged MyoD was shown to have full bioactivity by the criterion of converting non-myogenic fibroblasts to myocytes following infection with the lintiviral construct. A further variation has added an IRES GFP to enable us to identify expressing cells visually, and it also showed high bioactivity. The tagged MyoD protein was shown in IP-western analyses to bring down several expected associated proteins. We expect to begin mass spec analysis in the coming months on mammalian samples prepared from infected cells and from tissues of transgenics animals. An immediate question is whether we can detect associations that occur only on DNA (at so-called enhancesome complexes) from associations of the same proteins that do not depend on DNA (heterodimerization, for example).

173. Developing genomic DNA as a comprehensive cohybridization standard for use in microarray gene expression measurements

Richele M. Gwirtz, Brian A. Williams

Standardization of gene expression measurements on spotted microarrays is accomplished by ratiometric quantitation. This is necessary because the mass of hybridizable material deposited on each spot is not uniform. In practice, ratiometric standardization is accomplished by simultaneous co-hybridization of the array to two samples of fluorescently labeled nucleic acid. The general form of this ratio is that the numerator measurement contains fluorescent intensity for the experimental sample, while the denominator measurement contains fluorescent intensity for a reference sample. Subsequent comparison of multiple samples is accomplished through the common denominator RNA preparation. In any given comparison of two cell populations or experimental conditions, a number of genes will be expressed in both cell types, and will yield reliable ratiometric measurements. However, ratiometric measurements are problematic for genes that are not expressed in the reference population (the denominator measurement). As the denominator measurements become increasingly small, the ratiometric values for the gene in question become disproportionately large and unstable.

To address the various shortcomings of RNA "standards," we are testing genomic DNA as a comprehensive microarray cohybridization standard. This standard performs three functions: 1) Since genomic DNA includes all genes present in the mouse genome, it provides universal coverage of the spots on the microarray, and should avoid the problem of highly unstable denominator intensity figures in computed ratios; 2) Since the vast majority of genes are represented at equimolar concentrations in genomic DNA, available sites for hybridization of labeled experimental sample will be relatively uniform and modest in intensity; 3) Genomic DNA is the same in sequence content from lab to lab and from prep to prep. RNAs are not.

For organisms with a relatively low genomic DNA complexity, such as *Arabidopsis thaliana or C. elegans*, or yeast, the signal expected per gene feature is expected to be robust, and we have verified that this is the case. However, for genomes of high complexity and repeat content, such as mouse and human, the signals are predictably lower. Nevertheless, after optimizing the fluorescent labeling protocol for sheared genomic DNA, we have shown that genomic DNA can be used as a reliable and reproducible "denominator" on a gene array composed of 14,000 70mer oligonucleotides. We obtain a spot coverage of greater than 95%, with median positive signals between 15 to 20 times greater than the median signal obtained from negative hybridization controls. The next step will be to move the universal DNA standard to third flour so that each experiment can deliver a direct ratio between two RNAs and universal normalization of those values to DNA. Since the concentration of each gene in DNA is known, the genomic denominator also offers the prospect of actually quantitating RNA in unknowns.

174. Defining myogenic determination and differentiation on a whole genome scale

*Brian A. Williams, Richel Gwirtz, Shuling Wang**

The immediate biological goal in this project is to define comprehensively the gene expression states in a model vertebrate developmental pathway. Within this goal is the aim of dissecting out responses to multiple signaling pathways that act to enhance or suppress myogenic differentiation. This pathway has three defined stable cell states linked by dynamic transitions: 1) Proliferating multipotential mesodermal precursor cells that can elect the myogenic pathway or several other possibilities; 2) These multipotential precursors can be stimulated to convert to "determined" myoblasts which are proliferating unipotential muscle precursor cells; 3) Myoblasts can then be triggered to differentiate, at which point they express the genes and cellular properties of a mature muscle cell or myocyte.

The second goal is to use the data obtained to help evaluate our suite of different "clustering" algorithms to determine which ones are most useful in which ways for analyzing large-scale data of this kind (see entry from Chris Hart *et al.,* in this section). A third goal is to use the data to advance our current model of the regulatory circuitry that controls this developmental pathway. Clustered expression data is one key input in defining the circuit, which is then joined by comparative genomic

DNA sequence data and protein:DNA binding studies (see entry of Tristan DeBuysscher).

Fluorescently labeled cDNA populations representing skeletal muscle cells during several time courses of differentiation have been co-hybridized to the arrays in the presence of cDNA from each of several "reference RNAs." Biologically relevant reference RNA sets include undifferentiated proliferating myoblasts (mono-potential muscle precursor cells) and multipotential mesodermal precursor cells whose possible developmental fates include muscle cells. The comparisons of muscle differentiation courses with different reference RNA sets is designed to expose groups of genes whose expression in muscle changes relative to each of the different reference cell states. Through use of various clustering methods we have identified components of a contractile apparatus regulon. Detailed kinetics reveal that this group is subdivided into kinetic classes, and the working hypothesis is that each of these groups depends differently on input signals (calcium dependent; FGF dependent; insulin dependent, etc.). These suggest, in turn, differential participation by specific transcription factors activated or repressed by each signal. The regulon was also shown to contain a number of expressed sequence tags representing previously unidentified genes in the mouse genome. To internally monitor the readout of these pathways dissected from each other, we created modified myogenic cell lines that allow us to measure the activities of individual transcription factors that we know are important in our system (MyoD family sites; MEF2 sites) to assist in attributing readout at natural complex genes. This information, is then used in conjunction with computational tools to identify and cataqagorize the *cis*-acting regulatory domains that drive genes within the regulon. Other groups of genes that belong to novel clusters have been itentified and highlighted for further study, including some whose expression precedes any previously known regulator in the myogenic differentiation pathway, making them candidates for hitherto unknown up-stream regulatory functions.

**University of California, Irvine*

175. Comparative sequence analysis in vertebrate genomes: Seqcomp, FamilyRelations and MUSSA

*Tristan DeBuysscher, Nora Mullaney**

Comparative sequence analysis in our lab is based on Seqcomp and FamilyRelations tools, developed by C. Titus Brown in Dr. Eric Davidson's lab and Tristan De Buysscher in our lab. These tools (described in detail and available for use at http://family.caltech.edu) were created to help identify regions of DNA that are high quality candidates to function as *cis*-regulatory modules for nearby genes. They are based the straightforward expectation that more highly conserved domains of DNA sequence between two genomes that are at auspicious evolutionary distance from each other are good candidates for functional importance. Specifically, we expect some conserved domains located outside RNA coding regions of genes to regulate transcription by acting as promoters, enhancers, silencers, and locus control domains. Our work focuses on mammalian and, more recently, multiple nematode species with mouse and *C. elegans* as the respective reference model organisms.

The full analysis and feature set of Family Relations is restricted to two-way comparisons, plus a limited ability to handle three species. Our recent work focuses on the problem of many-genome comparisons, where we hope to gain further resolving power together with knowledge about genomes evolve in noncoding domains. The tool developed to view and analyze three and more genome comparisons we call MUSSA. It uses a recursive transitivity finding algorithm to analyze multiple Seqcomp files into an arbitrary N-way analysis. A GUI has been developed to allow biologists to navigate the results of a N-sequence analysis using a histogram of region conservation, graphical overview of the links of highly conserved region in the manner of FR, plus tools for viewing the sequence of the conserved regions, performing simple DNA motif searches, and annotating results.

Current applications using MUSSA encompass both the myogenic regulatory pathway of interest to our lab and other genes for which we have sufficient (several tens of kilobases) orthologous sequence from multiple species. At present we are focusing on vertebrate genomes for one set of studies and, in separate but conceptually similar project, we are studying genes from four species of nematode worms in a collaboration with Hiroke Shizuya and Paul Sternberg. Since many-species comparative data over > 20kb regions are new, we need to answer some very basic questions: How much resolving power one can get with each additional genome being compared? How does the evolutionary distance between genomes affect resolving power? What fraction of conserved features found outside RNA coding regions contribute to transcriptional regulation of nearby genes? For example, ongoing first-pass global comparison of mouse and human genomes is revealing far more non-coding sequence similarity than most investigators expected. A basic aim in the studies for which we developed MUSSA is to learn what fraction of these conserved domains, on average, govern transcription, what fraction have some other detectable function, and what fraction may be "accidents" of evolution.

Within a region that is broadly conserved among multiple species we find, as would be expected, that divergence at some positions is species specific, while at others it is common to an order or clade. For example, one analysis in progress compares 11 species from two of the proposed placental mammial clades (III: includes rodentia, primates; IV: includes cetartiodactyla, carnivora). We find features in which divergent base pairs within a large conserved block (several tens of base pairs) are conserved within rodentia alone (or one of the other orders), conserved only within one of the two clades, or may be totally divergent for each species. Current work involves quantifying these sequence divergences and relating them to suggested evolutionary distances between the species

and clades. This work is being used to refine candidate functional regions to a small set that can be experimentally tested for *cis*-regulatory functionality via lentiviral and BAC-mediated transgenesis.
*Undergraduate, Caltech

176. Identifying and testing candidate regulatory regions for genes of the myogenic regulatory circuit

Tristan DeBuysscher, Libera Berghella

Using the sequence comparisons and Family Relations tools, a dozen genes from the paraxial segmentation and myogenic regulatory circuits are being analyzed by mouse human comparisons. Mouse BAC sequences are from sequencing performed by collaborators and the DOE Joint Genome Institute. The BACs were originally obtained from the mouse BAC library made by Hiroke Shizuya and colleagues, with screening by Mai Wang of the Mel Simon genomics group at Caltech. The conserved noncoding candidate regulatory domains are amplified by PCR and cloned into reporter vectors designed for introduction into mice by transgenesis. Because the pathways in question are active at midgestation, the transgenics can be productively assayed ~ 9 days after injection, giving an acceptably rapid assay compared with germline transgenesis. This project has recently been further advanced by data obtained in collaboration with Dr. Eric Green (NIH Genome Institute) which is giving us a 6-genome deep comparison of several myogenic and somitogenic pathway genes. Initial functional analysis of myogenin via multigenom sequence comparison has uncovered a highly conserved element that appears critical for expression of myogenin in adult muscle.

177. MLX – A computational framework for machine learning and sata mining

Christopher Hart, Lucas Scherenbroic, Ben Bornstein*, Diane Trout, Joe Roden*, Barbara Wold, Eric Mjolsness***

Genome-scale analysis, including various genome sequence features, large scale RNA expression analysis, protein interaction maps, tissue arrays and complex collections of *in situ* images collectively present us with the problem of large amounts of data of diverse types. The need to perform many different computational analyses on these large datasets and then to view and integrate results from multiple analyses is a common problem in bioinformatics and for end-user biologists. We have therefore constructed a software architecture designed to provide easy access to many machine learning algorithms and techniques and to facilitate analysis of results derived from diverse algorithms. Using CORBA as the connection bridge, our suite of algorithms is available from several computational environments currently including mathematica, matlab, C/C++, python and Java. We have also implemented a fairly complete python application programmer interface (API), which provides a foundation for building novel analysis applications or can be used directly from the python interpreter as an interactive data analysis environment. Several of the lab's projects are now using and extending this framework.

The python API includes a powerful embeddable plotting tool, IPlot. IPlot provides for rapid construction of interactive 2D visualizations. In the case of microarray data analysis, it is critical to be able to quickly impose several data features onto a single plot. To accommodate this need, IPlot is designed to allow for the mapping of any data features onto any plot feature (i.e., color all points by cluster membership, Size all data points by p-values, point coordinates determined by PCA projection of a data vector, etc.). The other major feature of IPlot is to provide interactive "clickable" linkages to any information linked to a data vector, which is accomplished using the MLX architecture.

*Machine Learning Systems Group, Jet Propulsion Laboratories
**JPL/Computer Science Department, University of California, Irvine

178. Comparison of clustering algorithms for use in large-scale gene expression analysis

C.E. Hart, Diane Trout, Sagar Damle, B. Bornstein, J. Roden*, E. Mjolsness***

DNA microarrays and other large-scale gene expression analyses generate datasets of unprecedented size and complexity. Measurements for thousands of genes across tens to hundreds of tissue types, signaling stimuli, developmental time points, genetic variants, drug doses, and the like are now routine. A pivotal step in mining large-scale gene expression data is to group genes displaying similar expression profiles and/or to group samples that are most similar to each other. A growing repertoire of clustering algorithms can be used to reveal underlying structure in other kinds of large datasets and they are rapidly being adapted and fruitfully applied to large-scale expression data. As a group, these algorithms provide the essential computational infrastructure needed to extract patterns of co-expression that are often then used, alone or in conjunction with other data, to generate hypotheses about co-regulation, biological relatedness of samples, etc. Each algorithm makes different underlying assumptions about data structure and operates by a different mechanism. These differences are expected to – and do- interact with differently with data properties such as sample number, degree of sample similarity, gene number, and experimental noise. In addition, each algorithm requires the selection of distance metric and, for many, specification of the number of clusters and selection of initialization conditions. These choices also lead to differing outcomes. The end-user biologist is usually faced with a confusing array of possibilities and little guidance about relative strengths and weaknesses.

To measure and understand effects of dataset properties and algorithm properties we constructed a framework for comparing clustering results. We then made a systematic across several major algorithms using both real and synthetic microarray data. The comparative

tool framework includes a mechanism to quantitatively assess and visualize cluster overlap by generating receiver operator characteristic (ROC) curves. We also implemented modified normalized mutual information (NMI) and a linear assignment (LA) metrics (1) to interpret the degree of agreement and disagreement between different clusterings of the same dataset. In addition to these quantitive measures, we implemented interactive visualization tools (I-PLOT) to explore how different algorithms are organizing the data space. Thus far, we have implemented and compared: an Expectation Maximization (EM) algorithm searching for a mixture of Gaussians, K-means, phylogenetic clustering (Xclust) to which we have added a novel agglomeration step, and self organizing maps (SOMs).

Selection of the number of clusters sought is required at the outset in the majority of clustering algorithms, and we've found this choice has profound effects on the resulting clusterings that vary with the algorithm. Kmeans is exceedingly sensitive to selection of a correct cluster number (K), but correct K is almost never known to the user apriori when working with biological data. This fragility makes Kmeans, in our view, the least favored method among those tested. Determining K empirically by Monte Carlo cross-validation has been implemented for the statistically based EM algorithm, and we have found that EM itself is more robust to a range of K values than is Kmeans. Still different is XclustAgglom: When the K value is greatly over-estimated, it proved remarkably robust for giving the correct overall cluster structure in many cases. However, there are notable exceptions for certain dataset structures and selections of distance metric. We have also found that several of the algorithms are quite sensitive to initialization conditions (K means is also relatively fragile in this aspect). As one might expect, most of the algorithms do well with clusters that are well separated and relatively large in size. While this is useful at some level, a feature of most real biological datasets is the existence of relatively small important clusters, and of functionally important clusters that are not greatly separated from all other data vectors. We are currently addressing these specific issues.

*Machine Learning Systems Group, Jet Propulsion Laboratories

**Department of Computer Science, University of California, Irvine

Reference

(1) Forbes, A.D. (1995) *J. Clinical Monitoring* **3**:189-206.

179. pMesogenin1 regulates paraxial mesoderm specification and body segmentation in *Xenopus*

Jeong Kyo Yoon, Shuling Wang, Randall T. Moon***

Paraxial mesoderm in vertebrates gives rise to all trunk and limb skeletal muscles, the trunk skeleton, and portions of the trunk dermis and vasculature. A defining characteristic of all vertebrates is metameric segmentation of musculoskeletal and peripheral nervous systems. This body plan arises from primary segmentation of the paraxial mesoderm into tissue blocks called somites. The bHLH class gene we have named *pMesogenin1*, (isolated earlier as the E-protein heterodimerization partner from a two-hybrid screen in yeast), is specifically expressed in presomitic mesoderm in the as yet unsegmented block of paraxial mesoderm in mouse and in *Xenopus*. A striking feature of *pMesogenin1* expression is that its expression terminates abruptly in the presomitic mesoderm shortly before formation of the next somite (somites are formed continuously in a rostral to caudal gradient). Its closest relatives, MESP1 and MESP2, are expressed just after pMesogenin1 is shut down and just before the next somite forms. Thus, the bHLH subfamily of pMesogenin and MESP1/2 collectively define discrete but highly dynamic prepatterned subdomains of the paraxial mesoderm. The pattern of expression for pMesogenin1 is consistent with a functional role in specifying one or more aspects of paraxial mesoderm phenotype, and in this project I set out to test this possibility using both gain-of-function and loss-of-function experimental designs. In collaboration with Randy Moon, I found that *pMesogenin1* from either mouse or frog can efficiently drive non-mesodermal cells to assume a phenotype with molecular and cellular characteristics of early paraxial mesoderm. The assays were performed in *Xenopus* embryos or embryo explants that had been injected in early cleavage stages with RNA encoding pMesogenin1 or control RNAs of interest. Among genes induced by added *pMesogenin1* is *XWnt-8*, a signaling molecule that induces a similar repertoire of marker genes and a similar cellular phenotype. Additional target genes induced by *pMesogenin1* are *ESR4/5*, regulators known to play a significant role in segmentation of paraxial mesoderm. *pMesogenin1* differs from other known mesoderm inducing transcription factors because it does not also activate a dorsal (future axial) mesoderm phenotype, suggesting that *pMesogenin1* is involved in specifying paraxial mesoderm. In the context of the intact frog embryo, ectopic *pMesogenin1* also actively suppressed axial mesoderm markers and disrupted normal formation of notochord. In addition, we found evidence for cross-regulatory interactions between *pMesogenin1* and T-box transcription factors, a family of genes normally expressed in a broader pattern and known to induce multiple types of mesoderm. Based on our results and results from prior studies of related bHLH genes, we propose that *pMesogenin1* and its closest known relatives, *MesP1/2* (in mouse) and *Thylacine1/2* (in *Xenopus*), comprise a bHLH subfamily devoted to formation and segmentation of paraxial mesoderm.

To further define the function of pMesogenin1 in paraxial mesoderm formation, I created a germline deletion of mouse *pMesogenin1* with the help of Shirley Pease and her colleagues in the Transgenic Mouse Facility. *pMesogenin1* homozygous knockout embryos show a complete failure of trunk and tail somite formation and segmentation of the body trunk and tail. Although several genes, including members of the famous Notch/Delta

intercellular signaling apparatus, have previously been shown to partially disrupt segmentation, none have a phenotype of complete blockade, as is seen in the pMeosgenin1 animals. At the molecular level, the phenotype features dramatic loss of expression for components of the *Notch/Delta* pathway and the oscillating "somitic clock" genes that are thought to control segmentation and somitogenesis. The presumptive paraxial mesoderm also fails to execute patterning and specification steps, leading to a complete absence of all trunk paraxial mesoderm derivatives which include skeletal muscle, vertebrae and ribs. We infer that *pMesogenin1* is an essential upstream regulator of trunk paraxial mesoderm development and segmentation in vertebrates.

**University of California, Irvine*
***Howard Hughes Medical Institute, Dept. of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195*

180. Isolation of target genes for pMesogenin1

*Jeong Kyo Yoon, Jason Chua**

Functional analyses suggest that pMesogenin1 is a major regulator of paraxial mesoderm development and segmentation, based on mouse knockout and frog gain of function studies. We postulate that it functions at the molecular level, as do other bHLH proteins, as a sequence specific DNA binding protein. Based on precedents from other bHLH genes that have been studied in detail, it could act as a repressor, as an activator (the most commonly reported activity for these regulators), or as either one, depending on context in a given target gene. This raises the question of what genes are direct downstream targets of pMesogenin1 regulation and which genes are indirectly regulated by it. To address these questions, we generated recombinant DNA constructs that encode chimeric proteins in which the ligand binding domain of either glucocorticoid receptor (GR) or estrogen receptor (ER) was fused to the carboxy terminus of pMesogenin1 protein. Normally, native GR or ER function as transcription factors in the presence of their respective ligands, but they are inactive in their absence. When no hormone is bound, they are complexed with heat shock protein (HSP). These complexes are transcriptionally inactive and are localized mainly outside the nucleus. However, upon exposure to their corresponding hormones, GR and ER are released from their HSP complexes, relocalize and function in the nucleus. Subsequently the hormone-activated native ER and GR activate their transcriptional target genes by binding to specific DNA sequences via their DNA binding domains. Similar to native GR or ER proteins, it has previously been shown that chimeric proteins containing the hormone binding domains (but not the ER or GR AND binding domain) reside in the cytoplasm, and can be translocated into the nucleus upon hormone treatment. Therefore, the functional activity of the chimeric protein as a transcription regulator can be controlled in a hormone dependent manner.

To identify target genes, the chimeric protein will be expressed in both mouse cell culture and animal cap explants culture from *Xenopus* embryo. Then, RNA will be isolated from both naïve and cells/explants treated with hormone. An important option afforded by this hormone-dependent chimeric design, is separation of direct transcriptional targets from secondary and tertiary targets. Thus, if hormone is added in the presence of a protein synthesis inhibitor, direct targets can be up or down regulated. If hormone is added in the absence of a protein synthesis inhibitor, then primary, secondary and more indirect targets will all be affected. A microarray gene expression analysis will be applied to begin to identify genes that are either induced or repressed by the chimeric proteins in the context of appropriate cells from null animals.

**2000 SURF student, Biology, Caltech*

181. Expression and functional analysis of *FP1* during mouse development

*Jeong Kyo Yoon, Shuling Wang**

Expression of *T117* RNA is first detected in the rostral floor plate of the neural tube in e8.0 mouse embryos. Expression is extended more caudally as embryo develops. Expression of two previously known floor plate markers, *HNF-3beta* and *shh*, precedes that of *T117* in the floor plate, suggesting that *T117* may act downstream of *HNF-3beta* and *shh*. It is noteworthy, however, that unlike shh and HNF3beta RNAs, which are also expressed in other axial domains, T117 appears highly specific for the floorplate and for this reason has been named *Floorplate1 (FP1)*. The floor plate is a very interesting ventral structure in neural tube that produces important signaling molecules such as SHH and netrins function as patterning signals for of CNS neurons and for some other axial domains as well. In order to investigate the functional role of FP1 *in vivo*, both "gain-of-function" and "loss-of-function" studies are in progress. First, the FP1 bHLH coding region was engineered for ectopic expressed in the dorsal neural tube using either *Wnt-1* or *En-2* enhancer/promoters, and these have been introduced into fertilized eggs to generate transgenic embryos. The phenotypic consequences of ectopic expression, at least at the levels provided by these constructs were unremarkable. For a loss-of-function study, the *FP1* gene was disrupted in mouse ES cells and knockout mice were generated. The initial phenotypic analysis revealed that homozygous knockouts are also largely normal in development of the floorplate and of cells known to be affected by signals from the floorplate. However, a more detailed analysis will be needed to determine whether neuronal specification or pathfinding may have been altered in more subtle ways. We isolated a presumptive ortholog for FP1 has also been isolated from *Drosophila*, based on notable similarities in the bHLH domain protein coding sequences of the two genes. Preliminary *in situ* hybridization studies indicate that it is expressed in a specific subset of glial cells.

**University of California, Irvine*

182. Role of a homeogene, *msx-1*, in adult muscle regeneration

Libera Berghella

Regeneration of adult skeletal muscle after an injury or disease is understood to be mediated primarily by muscle satellite cells. In an intact muscle this population of cells is composed of rare, mononucleate cells located beneath the basal lamina of the mature fiber. In healthy adult muscle the satellite cells are mitotically quiescent. Muscle injury triggers their metabolic and mitotic activation. They emerge from G_o mitotic arrest, proliferate and concomitantly undergo a series of developmental changes. This pathway ultimately ends, for the majority satellite cell progeny, in differentiation and fusion to form new myofibers or differentiation coupled with fusion into existing fibers. However, some cells apparently have a different fate. Instead of differentiating as muscle fibers, they return to replenish the satellite pool in order to support future regeneration.

Biochemical and molecular analysis of muscle regeneration has, in the past, been hampered by the rarity of satellite cells and the absence of reliable molecular markers for these cells, especially when they are in the quiescent state. Prior work in this lab led to a method of studying gene expression in individual satellite cells over the timecourse of an activation response (Cornelison and Wold, 1997; Cornelison *et al*., 2000). Among the genes studied, the homeobox gene *msx-1* revealed a peculiar and interesting expression pattern, with transcripts being detectable only at extremely early timepoints (<30 minutes post-fiber isolation). This suggested that quiescent cells express *msx-1* but quickly downregulate its expression once they are activated. It is also known that forced expression of *msx-1* in cultured myoblasts inhibits differentiation and can suppress MyoD expression (Woloshin *et al*., 1995). Based on these and other preliminary results, our working hypothesis is that *msx-1* expression marks satellite cells that are in quiescence or are returning to it, and that it may be causal in suppressing expression of MRFs in quiescent satellite cells.

References

Cornelison, D.D.W. and Wold, B.J. (1997) *Dev. Biol.* **191**:270-283.

Cornelison, D.D.W., Rudnicki, M.A., Olwin, B.B. and Wold, B. J. (2000) *Dev. Biol*. **224**:122-137.

Woloshin, P. *et al*. (1995) *Cell* **82**:611-620.

183. Expression profiling of stem cell lines

*Libera Berghella, Sagar Damle, Brian Williams, Guilio Cossu**

In collaboration with the lab of Giulio Cossu at the University of Roma, we have initiated a large-scale gene expression characterization of cell lines isolated from the embryonic dorsal aorta of mice. The special interest in these cells comes from their capacity to differentiate into muscle, cardiocytes, bone or other derivatives when grown under various culture conditions. This mimics the *in vivo* potential of dorsal aorta multipotential cells implanted into a host animal via transfusion into the blood. Such transplanted cells can subsequently be found in mature muscle fibers, and in other tissues as well. Thus, the isolation and initial characterization of this cell line supports the hypothesis that multipotent mesenchymal cells may be present embedded in vascular endothelium (De Angelis *et al*., 1999). Using cDNA microarrays, made in the lab, I have begun to analyze the transcription program of these cells in comparison with more conventional myogenic cell lines that are believed to be unipotential and myogenic (C2C12, MM14) or multipotential (10T1/2), or totipotent (embryonic stem cells). Results have revealed genes expressed in common only with 10T1/2; or with 10T1/2 and ES cells, but not myoblast lines. Other genes are novel to one or both of the aorta lines, whose developmental potentials are only partially overlapping. Among genes in common with stem-like cells is CD34, a putative marker of stem cells in several other contexts. These results are being verified by independent methods, and the impact of the compound trichostatin (a global pharmacological modifier of chromatin access) are next to be tested and correlated with phenotypic impact.

**University of Roma, Italy*

Reference

De Angelis *et al.* (1999) *J. Cell. Biol.* **147**:869-877.

184. A new method for assaying gene expression

John Murphy, Barbara Wold, Brian Williams, David Tirell*, Mark Davis**

Gene microarrays are proving to be very powerful for large-scale gene expression analysis. However, they have significant shortcomings that we would like to bypass. They generally call for relatively large amounts of input RNA to achieve robust answers, and they are at their best with more abundantly expressed RNAs. They also have dynamic range limits that are considerably narrower than those of the RNA samples to be measured. In all their manifestations so far, they are semiquantitative, even under the best of circumstances. From an entirely different kind of methodology (multiplex single-cell RT-PCR (Cornelison and Wold, 1997) we know that being able to simultaneously monitor multiple genes in single cells gives a different and illuminating view of the gene expression combinatoric "states" that one cannot achieve using pooled cell samples. However the latter assays are thus far quite limited in simultaneous gene number (maximally seven simultaneous genes, thus far), and they also give little information about the quantity of each RNA type in an expressing cell. It is clear that in many biological settings, it would be highly desirable to get good quantitative measurements from just one or a few cells per determination for tens, hundreds, or even thousands of genes simultaneously.

To address this challenge, we are developing an approach that takes advantage of specifically designed families of chemical tags. Oligonucleotides, each designed to hybridize to a specific RNA specie of interest, are

synthesized such that each oligo carries a unique covalently attached peptoid (not peptide) tag with a unique mass. At the end of hybridization, chemical methods are used to separate hybridized oligos from those that do not react with a complementary RNA, and then tags are cleaved from their respective oligonucleotides. In these methods, the tags, rather than the RNAs or oligos, are ultimately counted. The first method of choice for analysis is mass spectrometry. We expect that this approach will permit direct RNA quantitation over several orders of magnitude, and it has the potential to begin with very small input RNA samples. A virtue of the design is that it uses hybridization in which all components are in solution phase, and these reactions are considerably better understood and more efficient than is nucleic acid hybridization in which one member is in solid phase, as is the case for microarrays. Multiple designs have been tested for linking the oligonucleotide moiety to the peptoid tag and robust strategy has been identified. Isotopic ratioing has also been owrked out to allow precise quantitation. Final proof of principle using well-defined RNA targets is in progress.

Division of Chemistry and Chemical Engineering

Publications

Cornelison, D.D.W., Rudnicki, M., Olwin, B. and Wold, B.J. (2000) MyoD deficient satellite cells in isolated fiber cultures are defective in MRF4 up-regulation and differentiation. *Dev. Biol.* **224**:122-137.

Csete, M., Walikonis, J. and Wold, B.J. (2001) Culture of mesodermal progenitor cells in lowered oxygen myogenic and adipogenic differentiation pathways. Accepted for publication.

Morrison, S.J., Csete, M., Grove, A.K., Melaga, W., Wold, B.J. and Anderson, D. (2000) Culture in lowered oxygen of isolated postmigratory neural crest stem cells reveals clonogenic sympathoadrenal lineage potential. *J. Neurosci*. **20**:7370-7376.

Studer, L., Csete, M., Lee, S-H., Kabbini, N., Walikonis, J., Wold, B.J. and McKay, R. (2000) Enhanced proliferation, survival and dopaminergic differentiation of CNS precursors in lowered oxygen. *J. Neurosci.* **20**:7377-7383.

Yoon, J.K. and Wold, B.J. (2000) p-Mesogenin a bHLH regulator of paraxial mesoderm specification and segmentation of *Xenopus*. *Dev. Biol*. **222**:376-391.

Yoon, J.K. and Wold, B.J. (2001) Mutation of mouse p-Mesogenin severely disrupts segmentation, somitogenesis, and formation of all paraxial mesoderm derivatives. *Genes Dev*. **14**:3204-3214.

Molecular Biology and Biochemistry

John N. Abelson
José Alberola-Ila
Giuseppe Attardi
David Baltimore
Pamela J. Bjorkman
Judith L. Campbell
David C. Chan
Stephen L. Mayo
James H. Strauss

George Beadle Professor of Biology, Emeritus: John N. Abelson
Research Scientists: Imre Barta, Tracy L. Johnson, Daniel Ryan, Scott W. Stevens
Assistant Biologist: Randall M. Story
Laboratory Staff: Joyce Kato, Santiago Laparra, Josephine Macenka, Blanca Mariona

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Summary: The focus of our laboratory has long been in understanding the mechanisms operative in gene expression. In particular we have been interested in RNA processing events that are required in the maturation of mRNA, tRNA and rRNA. In studying these events we employ biochemical and genetic approaches in the yeast *Saccharomyces cerevisiae*.

Eukaryotic genes contain introns that must be removed to generate the functional transcript. This is done by the process of splicing in which the intron is clipped out and the ends, the exons, are joined. Pre-mRNA splicing is at its heart likely to be RNA catalyzed but the reaction takes place on a large macromolecular machine, the spliceosome, which contains more than 80 proteins and five snRNAs found in the cell as ribonucleoprotein particles or snRNPs. There are five of these, U1, U2, U4, U5 and U6. Thus the task at hand is two-fold: to understand how the snRNAs interact with the pre-mRNA substrate to form the catalytic structure, and second to understand the role of the proteins. Progress has been made on both of these problems during the past year.

During the past four years Scott Stevens has taken the important step of developing a purification procedure for yeast snRNPs. His procedure involves one affinity step and a sedimentation step. In the process of characterizing the U4/U6 U5 triple snRNP, Scott discovered that at low concentrations of salt a significant portion of the snRNPs are found in the form of a penta snRNP complex containing all five snRNAs. We have purified this particle that contains all five of the spliceosomal snRNAs and 85% of all yeast proteins known to be affiliated with pre-mRNA splicing. This purified particle, or penta-snRNP, is active in pre-mRNA splicing when supplemented with soluble yeast factors, and the particle contains a U1 snRNP that is active in committing pre-mRNA substrates to the splicing pathway. The U4 and U6 snRNAs are base-paired, arguing that this particle is not an active spliceosome or a post-splicing complex of a lariat-intron. Furthermore, it has been shown that the penta-snRNP is likely to be the functional entity in splicing because the individual snRNPs do not reassociate in a mixing experiment. The protein composition of the particle was determined by mass spectrometry microsequencing. It contains all of the previously characterized yeast snRNP-associated proteins and in addition a set of proteins previously known be involved in splicing but not known to be snRNP associated. This fact alone implicates the penta-snRNP as the functional form of the spliceosome. Another set of proteins found in the particle provides evidence for links between reactions as diverse as RNA turnover, transcription and protein synthesis.

Tracy Johnson has continued with her work on the *trans*-splicing system to study RNA interactions in the spliceosome. In this system the 5' splice site, synthesized chemically, is joined in *trans* to the 3' splice site. Though *trans*-splicing is seen in other organisms such as worms and trypanosomes, it does not take place naturally in yeast and yet provides a very useful tool since one can incorporate powerful crosslinking agents into the synthetic 5' leader which is only 20 nucleotides long. Tracy has found that this process is slow *in vitro* and this has allowed her to see some novel crosslinks which provide an insight into early spliceosome assembly which have not been seen before in the natural *cis*-splicing systems which are faster. The most interesting of these is a 5' splice site U4 interaction that had not been seen before. Since this crosslink does not require an intact 3' splice site, it appears that the U4 snRNP interacts with the 5' splice site before the 3' splice site is recognized and fits very well with the apo-spliceosome role of the penta snRNP.

Perhaps the most interesting of the spliceosomal proteins are a set of RNA-dependent ATPases called "DEAD box" proteins. In some cases these proteins have been found to be RNA helicases and are implicated in the RNA rearrangements that occur during spliceosome assembly. Last year, Randy Story for the first time solved the complete structure of a DEAD box protein, in this case from the archaebacterium *M. jannaschii.* This structure will be an important tool in understanding the phenotypes of various mutations in conserved regions of the protein and as detailed in Story's report has already suggested modes of RNA interaction. Story is now focusing on a second RNA helicase DbpA, and *E. coli* protein that has a specific RNA substrate. Efforts are underway to crystallize the protein with its substrate, a fragment of 23S rRNA.

Publications

Johnson, T.L. and Abelson, J. (2001) Characterization of U4 and U6 interactions with the 5' splice site using a *S. cerevisiae in vitro trans*-splicing system. *Genes & Dev.* **15**:1957-1970.

Ryan, D.E. and Abelson, J. (2002) The conserved central domain of yeast U6 snRNA: Importance of U2-U6 helix I_a in spliceosome assembly. RNA. In press.

Ryan, D.E., Stevens, S.W. and Abelson, J. (2002) The 5' and 3' domains of yeast U6 snRNA: Lsm proteins facilitate binding of Prp24 protein to the U6 telestem region. RNA. In press.

Stevens, S.W., Barta, I., Ge, H.Y., Moore, R.E., Young, M.K., Lee, T.D. and Abelson, J. (2001) Biochemical and genetic analyses of the U5, U6, and U4/U6 U5 small nuclear ribonucleoproteins from *Saccharomyces cerevisiae*. *RNA* **7**:1543-1553.

Stevens, S.W., Ryan, D.E., Ge, H.Y., Moore, R.E., Young, M.K., Lee, T.D. and Abelson, J. (2002) Composition and functional characterization of the yeast spliceosomal penta-snRNP. *Mol. Cell* **9**:1-20.

Story, R.M., Li, H. and Abelson, J. (2001) Crystal structure of a DEAD box protein from the hyperthermophile *Methanococcus jannaschii*. *Proc. Natl. Acad. Sci. USA* **98**:1465-1470.

Assistant Professor: Jose Alberola-Ila
Postdoctoral Scholars: Gabriela Hernandez-Hoyos, Micheline Laurent, Daniela Leopoldt
Graduate Students: Susannah Barbee, Harry Green, Eric Tse
Undergraduate Students: Ayenna Bradshaw-Sydnor, Katherine Homan, Isaac See
Research and Laboratory Staff: Christie Beel, Chi Wang

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Summary: Genetic analysis of the signal transduction pathways involved in T-cell differentiation.

We are interested in studying the intracellular signaling pathways that regulate cell fate determination during T-cell development. Each developing T cell expresses a unique receptor (T-cell receptor) that interacts with molecules of the major histocompatibility complex (MHC) associated with short peptides. The TCR determines the antigen specificity of the T cell and is the most important element in the regulation of T cell development and function. Ligand occupancy of the TCR in immature CD4+CD8+ thymocytes may result in activation-induced apoptosis (negative selection), or in their survival (positive selection) and further differentiation into either CD4 or CD8 mature T cells. Though the existence of these selective events has been documented extensively, it remains puzzling that signals transmitted through the same receptor in seemingly identical cells may provoke either death or survival. Hence, the biochemical signals elicited by ligand occupancy of the TCR must either vary with the stimulating antigen, or must be interpreted flexibly in the context of other receptor-mediated signaling processes. Our research interests are focused in elucidating the role that different signal transduction pathways downstream the TCR play in the control of lymphocyte differentiation.

We have focused so far on the signaling requirements during thymocyte selection. We have shown that the Ras/MAPK pathway plays a pivotal and specific role during T cell development. In particular, expression of dominant-negative forms of Ras and Mek blocks positive selection of αβ T cells, while negative selection is not affected. We have also shown that the lineage commitment of the positively selected T cell is controlled by the intensity of the signal transmitted by the tyrosine kinase Lck. The research projects that we are pursuing build upon these systems and try to understand better the signaling networks that control the behavior of T cells.

185. Analyses of Ras effector pathways necessary for positive selection

Micheline N. Laurent, Katherine Homan

The Ras family of GTP-binding proteins is important in the regulation of many different activities within many diverse cell types, including choices made by T cells during their development. We have previously shown that the expression of a dominant-negative form of Ras (dnRas) in developing murine thymocytes is sufficient to block positive selection, such that the number of mature T cells is significantly reduced. Similarly, blockade of the Ras effector MAPKK also inhibited positive selection. However, activation of the MAPK cannot rescue the phenotype of a dnRas mouse, suggesting that additional Ras effectors are required during positive selection.

To determine which additional effectors are necessary for positive selection, we are utilizing a series of well-described Ras effector mutants that contain point mutations made within the effector loop region of a constitutively active form of Ras. Each of these mutants stimulates only a specific subset of pathways downstream of Ras, thereby providing us with a method to study the effects of activating only particular signaling pathways. Using retroviral-mediated gene transfer, these mutants were introduced into fetal thymocytes derived from animals expressing the dnRas transgene. Infected thymocytes are allowed to develop in fetal thymic organ culture (FTOC) for approximately two weeks and subsequently analyzed for their ability to rescue the effects of a dominant-negative form of Ras. One of the mutants that has been tested enhances T cell development in the dnRas background. This mutant activates the MAPK cascade, as well as the Ral exchange factor Ral-GDS, suggesting that the activation of Ral signaling, may assist Raf-MEK-ERK signaling in promoting T cell maturation. Future experiments will address the role of this signaling cascade in Ras-mediated positive selection.

186. Ksr connects Ras to the MAPK cascade

Micheline N. Laurent

It is well established that the Ras-Raf-MEK-ERK cascade is necessary for proper thymocyte development. However, the details related to the regulation of this pathway are incomplete, particularly in regards to the intermolecular associations made both prior to, and following signal activation. Recently the novel molecules, the Kinase Suppressor of Ras (KSR), Connector Enhancer of KSR (CNK), and Suppressor of Ras 8 (Sur-8) were discovered based on their ability to regulate Ras signaling in invertebrate developmental model systems. To determine if these molecules perform a similar role during T cell development, these molecules were tested in a fetal thymic organ culture assay. Over-expression of KSR in fetal thymocytes partially blocked positive selection, a phenotype consistent with the inhibition of Ras signaling. CNK and Sur-8 did not have any significant effect on T cell development in our assay. To determine the structural elements of KSR necessary for these results, a series of KSR mutants were generated and tested. This analysis

revealed that the ability of KSR to interact with MEK was necessary for its observed effects in thymocytes. All other domains were dispensable, suggesting that the ability of Ksr to couple Ras to the MAPK cascade was critical for its effect on T cell development. In agreement with this observation, KSR over-expression in a thymoma cell line inhibits ERK activation, while CNK and SUR-8 had no effect in this assay. These results suggest that KSR may function as the main scaffolding protein connecting Ras to the MAPK cascade, while Sur-8 and CNK may connect Ras to additional pathways.

187. Crosstalk between Notch and TcR signals during T cell development

Gabriela Hernandez-Hoyos, Ayenna Bradshaw-Sydnor, Chi Wang

Notch is a family of transmembrane receptors of which there are four genes in mammals: Notch1 to 4. When engaged by its ligand, Notch is cleaved, releasing an intracellular fragment that is translocated to the nucleus, where it activates transcription of various genes. Activation of Notch is involved in different cellular processes including differentiation and cell survival, in a variety of tissues. Work from various groups suggests that the Notch cascade is also involved in positive selection of T cells. Overexpression of activated Notch signaling can block CD4, or both CD4 and CD8 development, depending on which domains of the Notch intracellular fragment are overexpressed. It has been proposed that the Notch cascade blocks activation of DP thymocytes by dampening the TCR signal. The Ras/MAPK pathway is downstream of the TCR signal, is activated early on during positive selection, and is necessary for this process to occur. We have tested whether overexpression of activated Notch in a double positive cell line, affects the MAPK cascade, by measuring phosphorylation of the MAPK ERK1,2 using intracellular staining, following stimulation of cells transduced with bicistronic retroviral vectors co-expressing GFP and Notch. Although activated Notch reproducibly inhibits activation, as determined by inhibition of expression of the surface markers CD69 and CD5, it only marginally inhibits ERK phosphorylation, and only in response to weak stimulation (limiting concentrations of PMA). The partial inhibition of ERK phosphorylation requires the RAM and PEST, but not the TAD domain of Notch, and is also observed in resting, but not activated peripheral T cells. We conclude that while Notch strongly blocks upregulation of activation markers such as CD69, this effect is not due to a direct block of MAPK activation.

188. Characterization of negative regulators of the MAPK cascade

Susannah D. Barbee, Isaac See

Studies with dominant negative Ras and Mek have indicated that these molecules are extremely important for the process of positive selection. As we dissected these pathways, we generated mice expressing an activated form of Mek-1, Mek-1(Δ3,S118,S122) (Mek*), under the control of the lck proximal promoter. When expressed during development, however, this potent mutant induces the early formation of thymomas. Analyses of these thymomas showed that the downstream MAPKs Erk-1 and -2 are not activated in Mek* thymocytes, nor can they be activated by stimulation with phorbol esters. However, Erk-1 and -2 can be activated *in vitro* by Mek-1 following purification, suggesting a specific inhibitory factor may be present in the cell. Likewise, the other MAPK's SAPK and p38 are not and cannot be activated in Mek* thymocytes. Thus the constitutive activation of upstream members of the MAPK cascade likely results in the activation of inhibitory phosphatases that prevent activation of downstream MAPKs. We hope to uncover what phosphatases may play this role and discover how they affect developmental processes.

MAPK can be regulated by members of the family of dual-specificity protein phosphatases (DUSPs). Most of these phosphatases are nuclear molecules regulated at the level of transcription that can dephosphorylate tyrosine, threonine, and serine residues, although some family members may be regulated post-translationally. Using Entrez and BLAST searches, we have identified fourteen phosphatases classified as dual specificity phosphatases (DUSP 1-14) and two additional phosphatases that may be DUSP family members (TS-DSP1 and TS-DSP2): each contains the conserved HCXXRXXG motif and other sequence homology to known dual specificity phosphatases. Eight of these DUSPs have nucleotide sequences cloned from murine cDNA. The remaining sequences have only been characterized in humans or rats, but BLAST searches for each of these revealed highly homologous ESTs (90% or greater) from murine tissues. We have designed family member-specific PCR primers flanking the conserved DUSP domains with which to characterize the usage of DUSPs in the T lineage. By comparing the expression patterns of these proteins in wild-type C57Bl/6 thymocytes and splenocytes as well as those activated with PMA and Ionomycin (mimicking the crosslinking of TCR), we can begin to understand what DUSPs are important in T cells and how they are regulated. Comparison with DUSP usage in Mek* thymocytes may provide special insight into the role of DUSPs in thymocyte development.

We are also pursing an expression cloning system to identify Erk inhibitors, given the central role of Erk in thymocyte development. The system entails a DP immature thymocyte line that is stably transfected with a negatively selecting gene driven by a MAP kinase-responsive promoter. The cell line can then be infected with a retroviral cDNA library and selected for the survival in the presence of PMA stimulation: only cells that have been infected with Erk pathway inhibitors should be able to survive, and we can clone out resistance-conferring genes. We are constructing a mammalian expression vector with the HSV-TK gene driven by promoter elements from the Erk-responsive gene egr-1: a minimal egr-1 promoter sequence consisting of a 2-serum response

elements (SREs) and several ets motifs (for binding from the ets family of transcription factors) confers Erk-specific transcription of the reporter gene. We can then create stable transfectants in the 16610D9 cell line: a TCR+ DP thymocyte cell line obtained from S. Hedrick (UCSD) in which Erk is readily activated by treatment with PMA. Retroviral transduction with Erk pathway regulators should rescue cell survival in the presence of PMA and ganciclovir. Such genes may be critical for regulating Erk activation and feedback mechanisms in developing thymocytes and mature T cells

189. Real-time detection of MAPK activity using fluorescence resonance energy transfer

Harry Green

Previous work has shown that the activation of the Ras/MAP Kinase (MAPK) signal transduction pathway is necessary for positive selection of thymocytes. Positive selection is a process by which developing thymocytes receive signals through the TCR and determine whether they are viable and will live, or not viable and will die. Our purpose is to study the biochemical nature of Erk1/2 MAPK signaling within these thymocytes to gain insight into the mechanisms of positive selection. In particular, we wish to examine whether the cascade acts as a signaling rheostat, in which increasing exogenous signal begets increasing internal signaling in a linear fashion, or as an all-or-none response, in which internal signal is begotten only by reaching a threshold of exogenous signal. In an analogous fate determination system, data from *Xenopus* oocytes has implicated that an all-or-none mechanism of MAPK activation controls oocyte maturation induced by progesterone.

To perform these experiments we must analyze MAPK activity on a single-cell basis. Using the cyan and yellow variants of Green Fluorescent Protein (CFP and YFP, respectively), we are developing an assay that can detect MAPK activity on a single-cell level using single- and two-photon confocal microscopy and flow cytometry. Utilizing fluorescence resonance energy transfer (FRET), a phenomenon by which two fluorophores come into close proximity and the emission of one fluorophore (donor) causes the excitation of the other fluorophore (acceptor), we hope to be able to create a highly sensitive fluorescent protein substrate sensitive to Erk MAPK activation. Using short peptide sequences taken from known Erk substrates, we have created several gene fusion constructs containing CFP and YFP, with these peptides acting as phosphorylation sensitive substrates between the two fluorescent proteins. Several such constructs have been successfully tested *in vitro* and we are continuing with experiments in culture and *in vivo*.

190. Egr regulates positive selection upstream Id3

Eric Tse

Each developing thymocyte expresses a unique T-cell receptor (TCR), whose ligation triggers either apoptosis (negative selection) or survival and further differentiation (positive selection), and the process by which such antithetical fates are adopted in response to signals from a single receptor has remained a key question for immunologists. It has been previously shown that the Ras-ERK MAPK cascade plays a critical role in positive selection, but is dispensable for negative selection. Furthermore, recent studies have shown that activation of this cascade in thymocytes undergoing positive selection results in decreased DNA binding activity by the basic helix-loop-helix (bHLH) proteins E12 and E47; this reduction is effected by the upregulation of the helix-loop-helix (HLH) protein Id3, an inhibitor of E12 and E47. My studies address the role of the early growth response (Egr) family of immediate early genes as a potential downstream mediator of the Ras-ERK MAPK cascade in Id3 induction and, accordingly, in positive selection.

Our lab has generated transgenic lines of mice overexpressing NAB2, a suppressor of Egr activity, under the control of the lck proximal promoter. NAB2 expression elicits a mild reduction in the appearance of mature, positively selected T cells in the thymus; this reduction approaches 50% in OVA mice, whose exogenous expression of a specific class-I restricted TCR allows the study of a more uniform developing T-cell population. The ongoing characterization of these mice should reveal whether the expression of the NAB2 transgene is able to impair TCR-ligation-induced Id3 upregulation and, correspondingly, positive selection. I have also begun the study the effects of disrupting Egr activity with two other dominant negative Egr proteins, WT1:Egr1 and DEgr1; both retain the wild-type DNA-binding domain of Egr1 but lack the transactivation domain, and WT1:Egr1 also bears the transcriptional repressor domain of Wilms tumor-associated protein. In reaggregate fetal thymic organ cultures (rFTOCs), an *ex vivo* model of thymocyte development, thymocyte populations transduced with WT1:Egr1 show reduced numbers of CD4+CD8- cells with no significant change in the numbers of CD8+CD4- cells; thymocyte populations transduced with DEgr1 show a similar but weaker phenotype, consistent with its lack of exogenous repressor function. Currently, FTOCs with fetal thymocytes expressing transgenic TCRs are being performed to further characterize the effects of disrupting Egr function. These experiments should provide additional insight into the role of Egr family members as mediators of the Ras-ERK MAPK cascade in positive selection.

191. Role of PI3-K in T cell development

Susannah D. Barbee

Phosphatidylinositol 3-kinase (PI3K) has been implicated in the regulation of proliferation and survival of a number of cell types including lymphocytes. A heterodimer, PI3K activates the downstream molecule AKT that appears to be a bifurcation point that mediates many of PI3K's functions. The best-characterized role of Akt is the regulation of cell survival via multiple downstream effectors including Bad/Bcl-XL, NF-κB, GSK-3β, and forkhead transcription factors. Furthermore, PI3K may function downstream or in parallel with Ras.

The Tec family of kinases comprises another group of PI3K effectors, the most famous of which is Btk: The immune deficiency diseases XLA in humans and xid in mice have been mapped to defects in Btk signaling. The family members Itk, Rlk, and Tec are all expressed in the T lineage and appear crucial for TCR signaling in mature T cells. Genetic alterations of effectors including Akt and Itk have yielded a variety of phenotypes in mice that suggest a crucial role for PI3K in thymocyte development.

We have generated several lines of mice expressing p110ABD, the domain of PI3K p110a that mediates adaptor subunit binding, under the control of the lck proximal promoter. Expression of p110ABD induces constitutive Akt activity in thymocytes by sequestering the regulatory adaptor subunit p85, endogenous p110 exists as a highly unstable but catalytically active monomer. The thymii of p110ABD animals appear largely normal but with slight increases in the percentages and numbers the mature single positive thymocyte subsets. This effect is not due to alterations in the life span of DP thymocytes nor defects in negative selection, as assayed both *in vitro* (using OT-1 deletion assays) and *in vivo* (superantigens).

On the other hand, positive selection is enhanced by p110ABD expression: AND+ p110ABD CD4 SP thymocytes efficiently develop in a partially selecting H-2b/d background. The enhanced development of AND thymocytes in the presence of p110ABD expression is reminiscent of that observed with the hypersensitive Erk sevenmaker mutant, but we observe no potentiation of Erk signaling in p110ABD thymocytes. Transgene expression instead enhances Ca^{++} flux responses to CD3 crosslinking, reducing the concentration of antibody required to elicit maximal flux. This effect is likely mediated by a potentiation of the activity of Itk, in parallel to the described effect of PI3K on Btk activity in B cells.

192. Chemical-genetic analysis of the role of Lck in T cell signaling

Daniela Leopoldt

One of the first biochemical events after TCR stimulation is the activation of the src-family protein tyrosine kinase Lck. Studies from many laboratories, including ours, have shown that Lck plays a major role in T cell development and activation. However, the exact contribution of Lck to many of these processes remains badly defined, due in part to the limitations of the genetic and pharmacological approaches used so far. We are using a recently developed chemical-genetic approach to engineer a mutant Lck protein (Lck-T316G) that is supersensitive to highly specific low molecular weight kinase inhibitors. These cell-permeable drugs were specifically designed to inhibit Lck-T316G, but not wild-type Lck or any other protein kinase. Their use will allow us to perform a complete or partial functional knockout of Lck kinase activity at different stages of T cell development or immune responses.

Using the Cre/loxP-based gene targeting strategy, the T316G mutation will be knocked in exon 8 of the murine lck gene by homologous recombination in embryonic stem (ES) cells. In order to isolate a genomic lck clone from the target ES cell line we have screened a 129SVJ mouse library. Two partial clones were isolated and characterized. Clone 7-42 containing the first eight exons of the lck gene was used for the left (short) arm of the replacement vector after introducing the point mutation by site-directed mutagenesis. Additionally, a silent mutation introducing a BamHI site in exon 8 was performed. This restriction site will later be used in screening experiments to distinguish wild-type lck from targeted lck. Clone 7-111 covering intron 8 and downstream sequences including exon 12 was used for the right (long) arm of the replacement vector. Pluripotent murine ES cells will now be transfected with the NotI-linearized targeting vector for homologous recombination. Correctly targeted ES cells will be identified by Southern blot analysis and/or PCR and subsequently injected into blastocysts.

Thymocytes and peripheral T cells from wild-type and homozygous T316G mutant Lck mice will be harvested at discrete times during differentiation, treated *ex vivo* with different doses of selective and potent kinase inhibitors and tested for possible Lck-mediated signal transduction pathways. Generation of these mutant Lck-T316G mice will later allow us to test the hypothesis that Lck activity controls CD4/CD8 lineage commitment, i.e., the decision of a T cell to become either a CD4+ helper T cell or a CD8+ cytotoxic T cell.

193. Gata-3 regulates development of CD8 SP thymocytes

*Gabriela Hernandez-Hoyos, Michele K. Anderson**

The GATA-3 transcription factor is expressed in the thymus from embryonic day 12.5 onwards, throughout development, and in peripheral T cells. GATA-3 deficient mice die on embryonic day 12, making it impossible to study T cell development in these mice. Studies performed in Rag2-/- mice reconstituted with GATA-3-/-ES cells demonstrated that GATA-3 is essential for T cell development: development of GATA-3-/- T cells is blocked at the earliest CD4-CD8- double negative stage. In addition to its role during early T cell development, GATA-3 has been shown to play a central role in the differentiation of Th2 CD4 cells. However, the role of GATA-3 at later stages of T cell development, including positive selection, has not been addressed, mainly due to the lethality of the knockout mice. We have undertaken the study of GATA-3 development during thymocyte development using retroviral transfer of wild type and mutant GATA-3 constructs into fetal T cell precursors, and then analyzed their development in reaggregate fetal thymic cultures. Our results indicate that overexpression of wt GATA-3 blocks development of T cells at the earliest stages, when E14.5 fetal liver or fetal thymocyte precursors are used. To bypass this early block, we infected thymocytes at later stages of development, containing precursors past the β selection point (E15.5 and E16.5). When expressed at these stages, GATA-3 reduces

the output of T cells, however it increases the frequency of CD4 over CD8 mature single positive (SP) cells. The expression of a transactivation-deficient GATA-3 mutant (KRR), does not affect thymic output, but also promotes development of increased numbers of T cells into the CD4 lineage, while reducing the output of CD8 T cells. The effect of the KRR mutant nicely shows that the enhanced CD4 development does not result from death of other populations. It also indicates that the inhibition on growth or survival and the generation of CD4/CD8 SP induced by Gata-3 can be separated, and probably result from different domains and functions of Gata-3. To determine if Gata-3 can switch the development of would-be CD8 cells into CD4 cells, we also overexpressed Gata-3 in transgenic thymocytes expressing a class-I restricted TCR that induces development into the CD8 lineage. In this context, while Gata-3 completely inhibits development of CD8 cells, it does not promote development of CD4 cells expressing a class-I restricted TCR. Finally, to test if the effect of Gata-3 on CD4/CD8 differentiation is a normal function of endogenous Gata-3, we overexpressed a natural repressor of Gata-3 function, present in peripheral CD4 T cells, but not in thymocytes. Our results show that inhibition of Gata-3 function enhances CD8 development. Together with the observation that Gata-3 is naturally upregulated during positive selection of CD4 but minimally during selection of CD8 cells, our data indicate that the role of Gata-3 in positive selection is to inhibit CD8 development in developing CD4 cells. Whether it also plays a positively regulatory role in CD4 differentiation remains to be determined.

**Postdoctoral Fellow, Ellen Rothenberg's laboratory, Caltech*

Publications

Iritani, B.M., Alberola-Ila, J., Forbush, K.A. and Perlmutter, R.M. (1999) Distinct signals mediate maturation and allelic exclusion in lymphocyte progenitors. *Immunity* **10**:713-722.

Hernandez-Hoyos, G., Sohn, S.J., Rothenberg, E.V. and Alberola-Ila, J. (2000) Lck activity controls CD4/CD8 T-cell lineage commitment. *Immunity* **12**:313-322.

Bain, G., Cravatt, C.B., Loomans, C., Alberola-Ila, J., Hedrick, S.M. and Murre, C. (2001) Regulation of the helix-loop-helix proteins, E2A and Id3, by the Erk MAPK cascade. *Nature Immunol*. **2**:165-172.

Gong, Q., Cheng, A.M., Akk, A., Alberola-Ila, J., Gong, G., Pawson, T. and Chan A.C. (2001) Disruption of T cell signaling networks and development by Grb2 haploid insufficiency. *Nature Immunol*. **2**:29-36.

Grace C. Steele Professor of Molecular Biology: Giuseppe Attardi
Senior Research Associate: Anne Chomyn
Visiting Associate: Patricio Fernandez-Silva
Visitor: Nicola Raule
Senior Postdoctoral Fellow: Yidong Bai
Senior Postdoctoral Scholar: Petr Hájek
Postdoctoral Scholars: Jordi Asin-Cayuela, Jaehyoung Cho, Mark Helm, Miguel Martin-Hernandez, Jin Zhang
Graduate Student: Kathleen Richter
Undergraduate Students: Elisa Chan
Research and Laboratory Staff: Loredana Bellavia, Jennifer Fish, Catherine Lin, Maria Roldan-Ortiz, Priscilla Verduzco, Rosie Zedan

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Summary: In the past year, a substantial progress has been made in our analysis of the role of mitochondrial DNA mutations in human aging. A breakthrough was provided by the unexpected discovery that a homoplasmic (i.e., occurring in 100% of the mtDNA molecules in a cell) C to T transition at position 150, very close to an origin of mtDNA replication, occurs at a much higher frequency in leukocytes from a large group of centenarians (in ~17% of individuals) than in leukocytes from younger subjects (in ~3% of individuals) of an Italian population. Several additional observations have provided valuable insights into the nature and mechanism of this phenomenon. First, the same mutation was found in either homoplasmic or heteroplasmic form (i.e., coexistent with wild-type mtDNA) in skin fibroblasts from an independent group of subjects, with a tendency to increase in incidence with aging. Most significantly, in five longitudinal studies of fibroblasts, in which two samples were taken from the same individual at time intervals of 9 to 19 years, the mutation was shown to appear or to become more abundant in the cells, up to homoplasmy, in the second sample. This observation indicated clearly that the mutation is not necessarily inherited, but can arise *de novo* during life, its level changing with age to a different extent in different individuals, most probably under genetic control. That the same conclusion may apply to leukocytes was strongly suggested by the finding that the C150T mutation in these cells can also occur in heteroplasmic form, but, surprisingly, in different proportions in two subgroups of leukocytes, i.e., lympho-monocytes and granulocytes, the discordance between these two types of cells increasing dramatically with age.

As to the possible functional relevance of the C150T mutation, another surprising finding was that this mutation was consistently associated, in fibroblasts and in immortalized lymphocytes, with the disappearance of the normal origin of replication at position 151 and with the appearance of a functional novel origin at position 149. Whether this remodeling of the origin of mtDNA replication has implications for the regulation of mtDNA replication, and, in particular, for the copy number control, remains to be determined. An interesting possibility is that the somatic event(s) at or near position 150 leading to the appearance and/or amplification of theC150T transition may be a part of a remodeling of the mtDNA replication machinery, probably genetically controlled, that would accelerate mtDNA replication and compensate for the oxidative damage of mtDNA and its functional deterioration occurring in old age. The very probable involvement of a specific protein(s) in the phenomenon described above has prompted a search for such protein(s), which is presently under way.

Another area in which considerable progress has been made in the past year concerns the initial mitochondrial events occurring in human 143B osteosarcoma cells induced to apoptosis by the protein kinase inhibitor staurosporine. In particular, a penetrating analysis of the early decrease in endogenous respiration rate, preceding cytochrome *c* release, in staurosporine-treated 143B osteosarcoma cells, which had previously been discovered in our laboratory, has led to the surprising observation that this decrease in respiration rate is not caused by any changes in the activities of the respiratory enzymes. This finding has strongly suggested the occurrence of an apoptosis-induced alteration in the permeability of the outer mitochondrial membrane, presumably due to closure of the voltage-dependent VDAC anion channel, which would prevent oxidizable substrates and ADP from being taken up into mitochondria. This interpretation has been fully confirmed by another surprising finding, namely that of a striking hypersensitivity of the outer mitochondrial membrane in staurosporine-primed cells, as compared to naïve cells, to digitonin. This detergent, in fact, solubilizes the mitochondrial outer membrane of drug-treated cells, causing a dramatic loss of cytochrome *c* and respiration. The crucial observation here was the reversal by exogenous cytochrome *c* of the loss of respiration in digitonin-permeabilized cells, implying a removal of the block created by the closure of VDAC. These findings indicate the occurrence of two early changes in the outer mitochondrial membrane in staurosporine-treated cells, which take place long before cytochrome *c* is released from mitochondria in intact cells.

Still another area of research presently of great interest in our laboratory, in particular, that concerning the dynamic nature of the mitochondrial organization and its control by the nucleus, has seen significant progress in this past year. In particular, the role of the nuclear genes encoding the human proteins mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2), which control mitochondrial fusion,

has been investigated in various human cell lines exhibiting quite different mitochondrial morphology. A general correlation has been observed between the levels of expression of Mfn1 and Mfn2, as determined by Western blot analysis of cell lysates or of purified mitochondrial lysates using specific anti-Mfn1 and anti-Mfn2 antibodies, and the morphology of mitochondria, analyzed by confocal fluorescence microscopy. In particular, the highest level of expression of the two mitofusions was observed in HeLa cells, carrying mostly long filamentous mitochondria, and the lowest level in 143B osteosarcoma cells, carrying mostly punctate mitochondria. The evidence that the Mfn1 and Mfn2 proteins participate in the control of the mitochondrial organization of human cells has significant implications concerning the crucial role of the nuclear background in the intermitochondrial complementation of disease- or aging-related mutations of mtDNA.

194. Aging-related accumulation of a novel specific point mutation in the mtDNA control region from human leukocytes and skin fibroblasts

Jin Zhang, Jennifer Fish

Previous studies analyzing human fibroblast and muscle mitochondrial DNA (mtDNA) had revealed a large aging-dependent accumulation of tissue-specific point mutations in critical control sites for mtDNA replication. These mutations are the fibroblast-specific T414G transversion and the skeletal muscle-specific A189G transition and T408A transversion (1,2). These findings had suggested that the mtDNA aging-related sequence variations might play a role in longevity. It was of interest to investigate whether similar mutations occur in mtDNA of human leukocytes, since leukocytes are considered to be one of the most useful materials to monitor biological age and are the most easily accessible cells for biochemical and genetic analysis in the human body.

In a preliminary large-scale screening of leukocytes, mtDNA of lympho-monocytes and granulocytes from 43 centenarians aged 99 to 106 years and 78 control subjects 18 to 98 years old from an Italian population was analyzed for the possible presence of the main aging-dependent mutations previously identified in fibroblasts and skeletal muscle. For this purpose, the segment of the main mtDNA control region comprised between positions 21 and 719 (*Init-Tra-Rep*), which encompasses all the initiation sites for transcription and the primary and secondary origins of H-strand mtDNA synthesis, was PCR amplified, purified by agarose gel electrophoresis, and utilized as a template for allele-specific termination of primer extension, using appropriate primers (3). This analysis revealed the absence of the T414G and T408A mutations from all the 231 leukocyte mtDNA samples and the minimal presence of the A189G mutation.

To investigate the possibility that some point mutations accumulate with aging in leukocytes at different nucleotide positions from those previously found in critical control sites for mtDNA replication in fibroblasts and skeletal muscle, the *Init-Tra-Rep* PCR-amplified fragments from mtDNA of lympho-monocytes and granulocytes of two subjects 72 years old and four centenarians were cloned in *E. coli*, and 50 plasmids were isolated from each source. The plasmid DNAs were then subjected to PCR amplification of the two *Init-Tra-Rep* subfragments DLP4 (nt110-271) and DLP6 (nt260-540), and to a DGGE (Denaturing Gradient Gel Electrophoresis) assay, followed by a sequencing analysis, as previously described (1,3). This analysis indicated the presence of a C150T point mutation that was present only in plasmid DNAs from centenarian leukocytes, suggesting a possible aging-related mutation site in leukocytes. Due to these observations, we focused our attention on the C150T transition. A large-scale screening of the available leukocyte mtDNA samples for the occurrence of the C150T mutation was carried out by allele-specific termination of primer extension using the PCR-amplified *Init-Tra-Rep* fragment as a template and an appropriate primer. The results showed that the leukocyte mtDNAs exhibited the C150T mutation in homoplasmic or heteroplasmic form, and that the frequency of the mutation in homoplasmic form among the centenarians was very significantly higher than that among the younger subjects of the same population.

In other studies, we carried out a large-scale allele-specific termination of primer extension of fibroblast mtDNA from 32 single biopsy samples from individuals 20-week fetal to 103 years in age and from 11 pairs of longitudinal fibroblast samples. These were derived from a different set of individuals from those utilized for leukocyte analysis. The C150T mutation found in leukocytes was also observed in homoplasmic or heteroplasmic form in 28% of the single fibroblast samples, with an apparent tendency to increase with age. Most strikingly, among 11 pairs of longitudinal fibroblast samples, i.e., samples taken twice from the same individuals at time interval ranging from 9 to 19 years, five exhibited an increased accumulation of C150T mutation in the second sample. Taken together, these results clearly showed a new C150T aging-dependent transition in the mtDNA control region of both human leukocytes and fibroblasts, and strongly suggested a role of somatic events in the accumulation of the C150T mutation in mtDNA of both tissues.

References

(l) Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G. and Attardi, G. (1999) *Science* **286**:774-779.

(2) Wang, Y., Michikawa, Y., Mallidis, C., Bai, Y., Woodhouse, L., Yarasheski, K.E., Miller, C.A., Askanas, V., Engel, W.K., Bhasin, S. and Attardi, G. (2000) *Proc. Natl. Acad. Sci. USA* **98**:4022-4027.

(3) Michikawa, Y. and Attardi, G. (2002) *Meth. Mol. Biol.* vol. **197**: Humana Press Inc., p. 75-92.

195. Detection and identification of a remodeled origin of mtDNA replication associated with the C150T mutation

Jordi Asin-Cayuela, Jennifer Fish

Aging dependent point mutations within the main mtDNA control region have been identified in our laboratory in the past few years (1,2). Most recently, a C to T transition was found in human blood leukocytes at position 150 (3). Since the 150 position is very close to the secondary origins of Heavy-strand (H-strand) synthesis, we chose to investigate possible effects on initiation of replication associated with this mutation. Using primer extension, followed by separation of the extended products on a denaturating polyacrylamide gel, we compared patterns of nascent H-strands originating from wild type and mutant mtDNA samples. We found that wild-type and mutant cells exhibit the same pattern of nascent H-strands corresponding to the initiation sites previously described (4), with the exception of one type of nascent strands, which are clearly shifted. This change in pattern occurs in both human fibroblasts and immortalized lymphocytes, but not in granulocytes or lymphocytes from blood samples, implying that active cell division and mtDNA replication are necessary to observe the phenotype.

The position of the 5' end of the nascent H-strands was identified by sequencing the nascent H-strands. The bands of interest were cut and eluted from the primer extension gel, the corresponding DNAS being subsequently circularized and amplified by PCR. Each PCR product was then ligated into a cloning vector, and 25-50 clones were randomly selected for sequencing. The efficiency of this method was confirmed by testing a 75mer oligonucleotide of known sequence, which yielded 39 of 39 clones with the expected 5'-3'end sequence junction. Our sequencing results confirmed previously reported results indicating that position 191 is the primary origin of replication (5). However, in wild type cells, we found the secondary origins to be located at positions 151 and 146, rather than 147. Furthermore, in mutant cells, the origin at position 146 persists, but that at the 151 position is absent and has been replaced by a new origin at position 149. The fact that this pattern is both consistent in the two C150T transition-carrying cell types (fibroblasts and immortalized lymphocytes) and constant within a sample (100% mutant cells display only nascent H-strands corresponding to position 149) indicates that this change in replication origin is associated with the C150T mutation.

References

(1) Michikawa, Y. *et al*. (1999) *Science* **286**:774.
(2) Wang, Y. *et al.* (2001) *Proc. Natl. Acad. Sci. USA.* **98**:4022.
(3) Zhang, J. *et al.* Manuscript in preparation.
(4) Chang, D.D. and Clayton, D.A. (1985) *Proc. Natl. Acad. Sci. USA*. **82**:351; D. Kang *et al*. (1997) *J. Biol. Chem.* **272**:15275.
(5) Crews, S. *et al*. (1979) *Nature* **277**:192.

196. Investigation of proteins interacting with sites of human mtDNA main control region specifically targeted by aging-dependent accumulations of mutations

Jaehyoung Cho

To identify any protein factors involved in the tissue-specific aging-dependent mtDNA mutagenesis and copy number control, we have used several independent approaches. A search was carried out in HeLa cell for possible proteins interacting specifically with the mtDNA segments targeted by the mutations. In a previous report from this laboratory, the mtDNA molecules isolated from Triton X-100-lysed HeLa cell mitochondria were shown to form complexes with some proteins in their main control region, known as D-loop region (1). Therefore, we planned to purify the mtDNA-protein complex from a large volume of HeLa cell cultures, using sequential ultra-centrifugations in Metrizamide and CsCl gradients of Triton X-100 lysates of highly purified mitochondria, and then to investigate the identity of the proteins copurified with mtDNA using nanoelectrospray tandem mass spectrophotometry sequencing of protein bands from SDS-PAGE and a comparison with the available databases. In preliminary results, we have already isolated and identified some major proteins associated with HeLa cell mtDNA purified by the above method. The major protein bands are the mitochondrial single-stranded DNA-binding protein (mtSSB), the glutamate dehydrogenase and a homologue of the heat shock protein 70. All these proteins have been reported to be important molecules for mitochondrial function and mtDNA metabolism (2, 3). However, we also found that there are various minor proteins within the mtDNA-protein complex, and it is very possible that these minor proteins carry out important functions in the processes of tissue-specific aging-dependent mtDNA mutagenesis and/or mtDNA copy number control. Therefore, we are trying to identify these minor protein bands.

To investigate the DNA-binding proteins interacting with single-stranded segments of the mtDNA main control region more directly, we applied South-Western blotting using mitochondrial protein extracts and radio-labeled single-stranded DNA probes corresponding to selected segments of the main control region. It is very important to use single-stranded DNA molecules as probes because the main control region of mtDNA forms a very specialized triple-stranded structure (D-loop) involved in mtDNA replication and RNA-DNA hybrids involved in mtDNA transcription and RNA primer synthesis. This approach may give new useful information about the proteins participating in the tissue-specific aging-dependent mtDNA mutagenesis or mtDNA copy number control. There is no information from previous studies about single-stranded DNA-binding protein factors interacting with mtDNA with sequence specificity (3). In preliminary results, we have found eight candidate proteins of about 120, 55, 47, 39, 35, 30, 18 and 16 kDa in size, and all these proteins, except the 16 kDa mitochondrial single-stranded DNA-binding protein (mtSSB), show specific

single-stranded DNA binding activity. To elucidate the molecular nature of these proteins, we will carry out nanoelectrospray tandem mass spectrophotometry sequencing of the proteins eluted from the South-Western blots.

Sometimes, South-Western blotting analysis cannot detect some types of DNA-binding proteins, because one analyzes denatured and refolded protein molecules by this method. In fact, if some protein factors can interact with DNA only as homo- or hetero-oligomers, one cannot find such proteins using South-Western blotting analysis. The refolding efficiency is also a rate-limiting step determining the resolution for detecting specific protein molecules. Therefore, it will be necessary to apply a gel mobility shift assay system to analyze the interaction between intact mitochondrial extract proteins and radio-labeled single-stranded DNA probes corresponding to selected segments of the main control region. Also in this investigation, to elucidate the molecular nature of a candidate DNA-binding protein(s), nanoelectrospray tandem mass spectrophotometry sequencing of the proteins eluted from excised gel-shifted bands will be carried out.

After cloning the cDNAs for the candidate protein factors using the information provided by the mass spectrophotometry approach, we will determine the transcription patterns of the genes in various human tissues, in order to investigate whether there is any correlation between the expression of the proteins and the tissue-specific accumulation of mtDNA point mutations.

References

(1) Albring, M. *et al*. (1977) Association of a protein structure of probable membrane derivation with HeLa cell mitochondrial DNA near its origin of replication. *Proc. Natl. Acad. Sci. USA* **74**:1348-1352.

(2) Kaufman, B.A. *et al.* (2000) In organello formaldehyde crosslinking of proteins to mtDNA: Identification of bifunctional proteins. *Proc. Natl. Acad. Sci. USA* **97**:7772-7777.

(3) Moraes, C.T. (2001) What regulates mitochondrial DNA copy number in animal cells? *Trends Genet.* **17**:199-205.

197. Nuclear genes controlling morphology of human mitochondria

Petr Hájek, Ansgar Santel[1], Margaret T. Fuller[1]

Mitochondria are dynamic structures that can rapidly fuse or divide. This process is a prerequisite for intermixing of the mitochondrial DNA (mtDNA) and/or its products, and for functional intermitochondrial complementation of mtDNA mutations. Thus, the control of mitochondrial fusion and division may play an important role in the distribution of mutant mtDNA during aging-dependent accumulation of point mutations and in disease.

Mitochondrial fusion and division are regulated by several proteins currently being identified in different organisms (1,2). The human mitofusins Mfn1 and Mfn2 are thought to participate in mitochondrial fusion, while the dynamin-related protein, Drp1, has been proposed to be a part of the mitochondrial outer membrane division apparatus. Thus, mitofusins and Drp1 may play a role in opposite processes that control mitochondrial morphology.

The possible dependence of mitochondrial morphology on the endogenous expression of the genes encoding these proteins was investigated in various human cell lines. Confocal fluorescence microscopy of live cells stained with tetramethylrhodamine methyl ester perchlorate or MitoTracker red was used to analyze the mitochondrial morphology. This technique revealed that the cell type-dependent mitochondrial morphology varied from mostly punctate mitochondria in 143B osteosarcoma cells, through mostly short rod-like mitochondria in HepG2 hepatoma and A549 lung carcinoma cells, to mostly long filamentous mitochondria in HeLa cervical adenocarcinoma cells. Northern blot analysis of total RNA prepared from various cell types revealed no correlation between mitochondrial morphology and expression of the endogenous Drp1, Mfn1 and Mfn2 mRNAs.

Confocal immunofluorescence microscopy of fixed cells stained with anti-Mfn1 antibody and Western blot analysis of subcellular fractions using anti-Mfn1 and anti-Mfn2 antibodies showed that Mfn1 and Mfn2 are localized only to mitochondria. The relative levels of the Mfn1 protein in various cell lines were determined by Western blot analysis of whole cell lysates and of purified mitochondrial lysates. The endogenous Mfn1 protein was most abundant in HeLa cells, but it was reduced by 30 to 60% in other cell types analyzed (143B, HepG2, A549). Western blot analysis of the Mfn2 protein was performed on purified mitochondria lysates, since this protein was not detectable in whole cell lysates. The Mfn2 protein levels of purified mitochondria were normalized to the mitochondrial marker Hsp60. These experiments revealed a cell type-specific correlation between mitochondrial morphology and endogenous level of the Mfn2 protein. The highest expression level of Mfn2 was found in HeLa cells, a medium expression level, in HepG2 and A549 cells, and the lowest expression level (about one-fifth of that in HeLa cells), in 143B cells. These results strongly suggest that mitofusins, and particularly Mfn2, participate in the establishment of filamentous mitochondrial morphology, by promoting mitochondrial fusion. Furthermore, the anti-Mfn2 antibody immunoreactive band detected on Western blots of 143B mitochondrial lysates migrated in PAGE faster than that from HeLa cell mitochondria. The possible structural basis of this electrophoretic mobility change of the Mfn2 protein is currently being investigated.

Although confocal immunofluorescence microscopy confirmed that a fraction of Drp1 co-localizes with mitochondria, Western blot analysis of subcellular fractions showed that the majority of Drp1 is cytosolic, and that only a very small fraction of this protein is associated with mitochondria. Because of these findings, the connection, if any, between mitochondrial morphology and Drp1 protein expression is so far not clear.

The above reported observation that 143B cells exhibit a low level of Mfn2 expression, when compared to HeLa cells, may also explain the strikingly different results concerning the frequency of intermitochondrial complementation of mtDNA mutations observed in Attardi's and Hayashi's laboratories using 143B cells and HeLa cells, respectively (3-6).

[1]*Stanford University School of Medicine, Stanford, CA*

References

1. Santel, A. and Fuller, M.T. (2001) *J. Cell Sci.* **114**:867-874.
2. Van der Bliek, A.M. (2000) *J. Cell. Biol.* **151**:F1-F4.
3. Yoneda, M. *et al.* (1994) *Mol. Cell. Biol.* **14**:2699-2712.
4. Enriquez, J.A. *et al.* (2000) *J. Biol. Chem.* **275**:11207-11215.
5. Ono, T. *et al.* (2001) *Nature Genet.* **28**:272-275.
6. Attardi, G.A. *et al*. (2002) *Nature Genet*. 3**0**:360.

198. Mitochondrial outer membrane permeability change and hypersensitivity to digitonin early in staurosporine-induced apoptosis

Shili Duan, Petr Hájek, Catherine Lin, Soo Kyung Shin, Giuseppe Attardi, Anne Chomyn

We have shown that the apoptosis inducer staurosporine causes an early and progressive significant decrease in the endogenous respiration rate in intact 143B.TK$^-$ osteosarcoma cells. This contrasts with the marginal decrease in the activity of the last enzyme of the respiratory chain, cytochrome *c* oxidase, in the first eight hours of staurosporine treatment. The decrease in the endogenous respiration rate precedes the release of cytochrome *c* from mitochondria, is not caspase-dependent, is not a result of the opening of the permeability transition pore, and is not blocked by overexpression of the gene for the anti-apoptotic protein Bcl-2. The latter finding suggests that Bcl-2 acts downstream of the perturbation in respiration. Furthermore, we have obtained evidence indicating that the cause of the respiration decrease is an alteration in the permeability of the outer mitochondrial membrane, presumably due to closure of the voltage-dependent anion channel, which prevents oxidizable substrates from being taken up into mitochondria. Further analysis of respiration has led to the surprising finding that staurosporine treatment of 143B.TK$^-$ cells causes their outer mitochondrial membrane to become hypersensitive to digitonin. This change results in a nearly total premature release of cytochrome *c* from mitochondria of digitonin-permeabilized cells. Most significantly, Bcl-2 overexpression prevents the staurosporine-induced hypersensitization of the outer membrane. Our experiments have thus revealed two early changes in the outer mitochondrial membrane, which take place long before cytochrome *c* is released from mitochondria in intact cells.

199. New cellular models for the study of mitochondrial diseases

Miguel Martin-Hernandez

Previous work in our laboratory had addressed the question of the pathogenetic mechanisms of different mitochondrial DNA (mtDNA) mutations involved in mitochondrial diseases. Important advances were made, showing that mtDNA point mutations are related to several mitochondrial dysfunctions that play a key role in the cellular pathological phenotype (1,2). However, further work needs to be done in order to increase our knowledge of the relationship between the mutant mtDNA content and the threshold for the pathological phenotype in these diseases, as well as of the role of the nuclear background in these events.

For this purpose, a rho 0 (mtDNA-less) cell line derived from the SH-SY5Y neuroblastoma cell line has been constructed to be used for mitochondria-mediated transformation, in order to develop new cellular models of mtDNA-linked diseases. These neuroblastoma cells, as well as the rho 0 derivative cell line, have the ability to differentiate, after an appropriate chemical stimulus, into neuronal cells, giving us a useful tool to mimic the conditions *in vivo*.

Neuroblastoma cells differentiated after retinoic acid treatment have been characterized by phase-contrast microscopy and immunofluorescence techniques in order to obtain the right conditions for further experiments using these differentiated cell lines.

Rho 0 neuroblastoma transformation experiments, using cell lines carrying the mitochondrial tRNA$^{Leu(UUR)}$ A3243G point mutation associated with the MELAS encephalomyopathy syndrome (constructed in our laboratory), are being performed in order to develop cellular models that carry the mtDNA mutation in a neuroblastoma nuclear background able to be induced to differentiate. We plan to use this system to characterize the pathogenetic mechanism of the MELAS mutation. In particular, we want to address the questions of the relationship between mtDNA mutation content and phenotype threshold for the different mitochondrial dysfunctions related to the MELAS syndrome, and of the role of the nuclear background, by taking advantage of the differentiated transformants as the model nearest to the *in vivo* situation.

References

(1) Chomyn, A., Enriquez, J.A., Micol, V., Fernandez-Silva, P. and Attardi, G. (2000) *J. Biol. Chem.* **275**:19198-209.
(2) Enriquez, J.A., Chomyn, A. and Attardi, G. (1995) *Nature Genet.* **10**:47-55.

200. A post-transcriptional modification enzyme imports structural information for tRNA into mitochondria: N^1-A9 methyltransferase confers secondary and tertiary structure to mt-tRNA^{Lys}

Mark Helm

The evolutionary loss in eukaryotic cells of mitochondrial (mt) tRNA genes and of tRNA structural information in the surviving genes has led to the appearance of mt-tRNAs with highly unusual structural features, sometimes referred to as "bizarre" tRNAs. One such bizarre tRNA is the human mt-tRNA^{Lys}, which relies on post-transcriptional base modification to achieve correct three-dimensional structure. It was previously shown that the *in vitro* transcript of human mt-tRNA^{Lys} adopts a particular, non-cloverleaf structure, when devoid of modified bases, while the native, fully-modified tRNA shows the expected cloverleaf structure (1). Furthermore, a methyl group at position N^1-A9, introduced chemically in an otherwise unmodified mt-tRNA^{Lys} transcript, was found to induce a stable cloverleaf conformation, raising the question of how the specific methyltransferase recognizes the unmodified transcript (2).

In order to shed light on this unusual case of tRNA maturation, *in vitro* tRNA modification systems contained in protein extracts from both highly purified HeLa cell mitochondria and HeLa cell cytosol were first analyzed and compared, and then used to study the mt-tRNA^{Lys}. The initial screening for modification activities was carried out using, as substrates, unmodified *in vitro* transcripts of tRNA genes with well-characterized structures, namely yeast cytosolic tRNA^{Phe}, human cytosolic tRNA^{Lys}-, and human mt-tRNA^{Ile}. The presence of 9 and 11 modification activities in the mitochondrial and cytosolic protein extracts, respectively, was detected, the mitochondrial extract including an N^1-A9 methyltransferase activity.

The conversion by post-transcriptional modification of unmodified, misfolded mt-tRNA^{Lys} to its cloverleaf structure was investigated in detail, with particular emphasis on the key role of N^1-A9 methyltransferase activity. The comparison of the level and kinetics of N^1-A9 methylation and other secondary modifications in the misfolded mt-tRNA^{Lys}, as compared to a cloverleaf-shaped structural mutant, engineered to adopt the tRNA^{Lys} cloverleaf structure without post-transcriptional modifications, suggested that the methylation of N^1-A9 in tRNA^{Lys} proceeds via a cloverleaf-shaped intermediate, which is present as part of a dynamic equilibrium. In conclusion, the mitochondrial protein extract contains an activity that stabilizes by secondary modification such a transient cloverleaf-shaped intermediate of mt-tRNA^{Lys}. Thus, countering the evolutionary loss of structural information in mt-tRNA genes, the mt-tRNA structure is maintained by a modification enzyme encoded in nuclear DNA. Ultimately, this represents the import of structural information for tRNA from the cytosol into mitochondria, in the form of a protein, a phenomenon similar to the example of the nuclear-encoded editing enzyme which converts the mt-tRNA^{Gly} into tRNA^{Asp}, in both aminoacylation and mRNA decoding specificity, in marsupials.

References

(1) Helm, M. *et al*. (1998) *Nucl. Acids Res*. **26**:1636-1643.
(2) Helm, M. *et al*. (1999) *Biochemistry* **38**:1338-13346.

201. Control of mTERF activity by larger size complex formation

Jordi Asin-Cayuela

The human mitochondrial transcription termination factor (mTERF) is a 39 Kda protein able to stop transcription at the boundary between the 16S ribosomal RNA and the tRNA$^{Leu(UUR)}$ mitochondrial genes (1), and is therefore believed to play a crucial role in the control of the expression of the mitochondrial ribosomal RNA genes. Although this protein contains three leucine zipper domains, it is known to bind to DNA as a monomer (2). Therefore, it has been proposed that its leucine zippers establish intramolecular interactions that would bring two basic domains together, enabling its interaction with DNA (2). Nonetheless, considering that leuzine zippers are protein-protein interaction domains, we explored the possibility of mTERF binding to other proteins. Gel filtration chromatography of an S-100 fraction from a HeLa cell mitochondrial lysate, followed by immunoblotting using an antibody against mTERF, clearly showed that mTERF elutes in two peaks, one at around 39 Kda and the other at around 110 Kda. Band-shift experiments, Western blotting of the shifted bands and heparin chromatography showed that the low molecular weight form (LMW), but not the high molecular weight form (HMW), binds to DNA. *In vitro* transcription termination activity tests showed that only the LMW form has transcription termination activity. These observations led us to conclude that the HMW form is an inactive form of mTERF, and the LMW form corresponds to the active form, as expected considering published results [2]. Both the characterization of the HMW form and the identification of the mechanism responsible for the transition between the two forms will be of paramount importance for understanding the control of mTERF activity.

References

(1) Kruse, B. *et al.* (1989) *Cell* **58**:391.
(2) Fernandez-Silva, P. *et al*. (1997) *EMBO J* **16**:1066.

Publications

Attardi, G. (2002) Role of mitochondrial DNA in human aging. *Mitochondrion*. In press.
Attardi, G., Enriquez, J.A. and Cabezas-Herrera, J. (2002) Intermitochondrial complementation of mtDNA mutations and nuclear context. *Nature Genet*. **30**:360.

Bai, Y., Hájek, P., Chomyn, A., Chan, E., Seo, B.B., Yagi, T. and Attardi, G. (2001) Lack of Complex I activity in human cells carrying a mutation in mtDNA-encoded ND4 subunit is corrected by the *Saccharomyces cerevisiae* NADH-quinone oxidoreductase (NDI1) gene. *J. Biol. Chem*. **276**:38808-38813.

Duan, S., Hájek, P., Lin, C., Shin, S.K., Attardi, G. and Chomyn, A. (2002) Mitochondrial outer membrane permeability change and hypersensitivity to digitonin early in staurosporine-induced apoptosis. In press.

Ferraro, E. and Attardi, G. (2002) Role of mitochondria in apoptosis of PC12 cells induced by staurosporine. In preparation.

Greco, Villani, G., Mazzucchelli, F., Bresolin, N., Papa, S. and Attardi, G. (2002) Marked aging-related decline in efficiency of oxidative phosphorylation in human fibroblasts. Submitted.

Guan, M.-X., Fischel-Ghodsian, N. and Attardi, G. (2001) Nuclear background determines biochemical phenotype in the deafness-associated mitochondrial 12S rRNA mutation. *Hum. Mol. Genet*. **10**:573-580.

Hájek, P., Villani, G. and Attardi, G. (2001) Rate-limiting step preceding cytochrome *c* release in cells primed for Fas-mediated apoptosis revealed by analysis of cellular mosaicism of respiratory changes. *J. Biol. Chem*.**276**:606-615.

Helm, M. and Attardi, G. (2002) A post-transcriptional modification enzyme imports structural information for tRNA into mitochondria: N^1-A9 methyltransferase confers secondary and tertiary structure to mt-$tRNA^{Lys}$. In preparation.

Michikawa, Y. and Attardi, G. (2002) Screening for aging-dependent point mutations in mtDNA. In: *Methods in Molecular Biology, vol. 197: Mitochondrial DNA – Methods and Protocols* (ed. W.C. Copeland), Human Press Inc., p. 75-92.

Michikawa, Y., Laderman, K., Richter, K. and Attardi, G. (2002) Role of nuclear background and in vivo environment in variable segregation behavior of the aging-dependent T414G mutation at critical control site for human fibroblast mtDNA replication. *Somat. Cell Mol. Genet*. **25**:333-342.

Zhang, J., Asin-Cayuela, J., Fish, J., Michikawa, Y., Bonafé, M., Olivieri, F., DeBenedictis, G., Franceschi, C. and Attardi, G. (2002) Strikingly higher frequency in centenarians and twins of mutation causing a remodeling of replication origin in leukocyte mtDNA. Submitted.

Professor: David Baltimore
Postdoctoral Scholars: Mark Boldin, Eric Brown, Huatao Guo, Alexander Hoffmann, Carlos Lois, Wange Lu, Mollie K. Meffert, Joel L. Pomerantz, Matthew Porteus, Xiao-Feng Qin
Graduate Students: Thomas Leung, Jeff Wiezorek, Lili Yang
Undergraduate Students: Elizabeth Hong
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Summary: Our laboratory currently explores six different areas: the control of NF-κB activation, the role NF-κB plays in various systems, the function of the *abl* gene, regulation of T lymphocytes, DNA recombination and repair, and development of lentivirus-based vectors.

The largest program in the laboratory involves study of the NF-κB transcription factor. Our major tool is a collection of mice in which we have knocked out one or more of the genes encoding proteins that are part of the NF-κB complex or control its activity. Using these mice and cells derived from them, we are studying the role of the individual proteins in controlling specific genes in various cell types. A major program involves using fibroblasts of defined genotype. We are also studying macrophage functions. At the level of transcriptional regulation, the questions we are investigating include whether the various NF-κB-related proteins have different functions and whether the sequences of the individual sites that bind NF-κB have regulatory relevance

NF-κB is found in high concentrations in neurons and, being present at synapses, is ideally positioned to serve a role as a mediator of synaptic activity. We are examining this possibility by using the knockouts and direct study of neuronal and synaptosomal preparations. We are also examining knockouts for behavioral defects.

There are many pathways for activation of NF-κB. We are studying a new one we uncovered involving the novel protein kinase TBK1. We are also examining the pathways of NF-κB and JNK activation using genetic methods in cell culture to determine which of the many postulated signaling pathways are critical to the activation process.

This laboratory has had a long-standing interest in the role of the *abl* gene in oncogenesis and in normal cellular physiology. We have shown that the closely related *arg* gene overlaps in its functions with *abl*, which had confused understanding of *abl*. The double knockout is an embryonic lethal. As a new approach, we have turned to *C. elegans.* This organism has only one *abl* gene, facilitating genetic studies. Our present results suggest that *abl* acts through an interaction with EPH receptors and we are investigating that possibility in detail.

Another interest in the laboratory has been in immunological tolerance and autoimmunity. In this regard, it is striking that mice lacking IL-2 or a functional IL-2 receptor accumulate activated T cells and develop autoimmunity, suggesting that the dominant role of IL-2 *in vivo* is to terminate T-cell responses and to maintain tolerance. To further study autoimmunity, we wish to create mice that express T-cell receptors known to be involved in autoimmune reactions. We are developing retrovirus-based vectors to facilitate this process and have had some success recently.

A further interest of our laboratory is the role of a group of large protein kinases in intracellular signaling processes. One is ATR—we have found that a knockout of the gene encoding this protein leads to very early embryonic lethality. We are investigating ATR in more detail through a conditional allele.

We are interested in the factors that limit the rate of homologous recombination between exogenously introduced DNA and the chromosomal DNA. To study this question, we have made cellular substrates that report homologous recombination and have characterized certain variables. We will extend this work by trying to increase rates of site-specific recombination events and to understand the genetic basis of the low rate in somatic cells. We are also investigating whether we can use mobile type II introns for gene transfer in mammalian cells. Mature neuronal cells do not divide again and thus there are no cell lines representing such cells.

A major success in the last year was to develop a lentivirus-based vector system that can bring genes into cells as transgenes. These transgenes are expressed stably over many generations of breeding and can be expressed in a tissue-specific manner. We are now investigating whether RNAi can be used to provide a lentivirus-based knockout system.

202. Lentiviral generation of transgenic mice

Carlos Lois

We have developed a system to generate transgenic animals by delivering genes via lentiviruses. Single-cell mouse embryos were infected *in vitro* with recombinant lentiviral vectors to generate transgenic mice carrying the green fluorescent protein (GFP) gene driven by a ubiquitously expressing promoter. Eighty percent of founder mice carried at least one copy of the transgene and 90% of these expressed GFP at high levels. Progeny inherited the transgene(s) and displayed green fluorescence.

Mice generated using lentiviral vectors with muscle-specific and T lymphocyte-specific promoters expressed high levels of GFP only in the appropriate cell types. We have also generated transgenic rats that express

GFP at high levels, suggesting that this technique can be used to produce other transgenic animal species. Integrated lentiviruses apparently are not subject to the efficient silencing that oncoretroviruses experience during embryonic development.

We are currently pursuing two extensions of this work: a) analysis of the mechanism that triggers silencing of oncoretroviruses; and b) generation of transgenic birds by lentiviral delivery.

Reference

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) *Science* **295**:868-872.

203. A systems analysis of NF-κB/IκB signaling: Genetics, computation, microarray

Alexander Hoffmann, David Baltimore

The transcription factor NF-κB regulates a surprising number of genes that fall into distinct functional categories: inflammatory and immune responses; cell survival and activation. Its activity is regulated in response to a variety of cell stimulatory signals, such as LPS, dsRNA, TNF, cytokines and growth factors. We are using a systems approach to understanding specificity in signal transduction and gene regulation.

NF-κB encompasses five gene products (p50, p52, RelA, c-Rel, RelB) that are capable of homo- and heterodimerization through the shared rel-homology domain (RHD). Cytoplasmic vs. nuclear localization of the resulting transcription factors is controlled by a family of three IκBs. Over the past few years, each of these genes have been deleted in mouse knockout strains. While interesting phenotypes have been described, we have documented extensive compensatory mechanisms within the NF-κB/IκB family.

Over the last two years we have generated multiple combination knockouts that have revealed additional functions for NF-κB in immune activation mechanisms, control of apoptosis and growth. Comprehensive panels of singly and multiply deficient cell lines allow addressing the questions of specificity in gene regulation. Such studies are being continued in a comprehensive manner utilizing gene chip technology to understand the specificity code in promoters for transcriptional control by NFκB/Rel dimers.

Nuclear localization of NF-κB is regulated by the action of three IkB isoforms. One of these, IκBα, is induced by NF-κB, thereby forming a negative feedback loop. Utilizing three cell lines, each deficient in two IκB genes we have constructed a computational model to describe the IκB-NF-κB signaling module. This model has revealed bimodal signal processing with respect to the duration of the stimulus. Our experiments have confirmed this and shown that bimodal temporal processing is a mechanism for generating differential gene expression responses with the same signal transduction pathway. Further exploration and extension of the computational model is a primary focus, and is complemented by experimental investigation of gene activation and cellular responses.

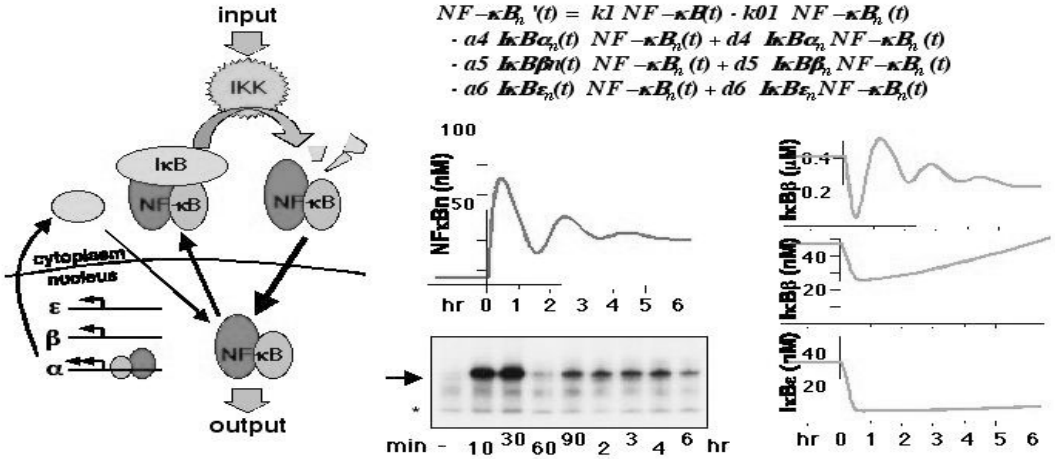

Fig. Computational Modeling of the IκB-NF-κB signaling module.

204. Cooperative and singular roles of ATR and ATM in DNA damage cell cycle checkpoint pathways

Eric J. Brown

ATR (ATM- and RAD3-related) is a high molecular weight PIK-related kinase that is the closest mammalian ortholog to central regulators of DNA damage responses in *S. cerevisiae* (MEC1), *S. pombe* (RAD3) and *D. melanogaster* (Mei41). In mammals, ATR shares function in DNA damage responses with ATM, another PIK-related kinase originally identified and cloned as the gene mutated in patients with ataxia-telangiectasia (A-T). To further examine the role of ATR in cell cycle checkpoint pathways, a cre/lox ATR-conditional mouse line was generated. Using ATR-conditional, ATM and ATR/ATM double knockout cells, we have shown that ATR cooperates with ATM in regulating ionizing radiation (IR)-induced signaling pathways that prevent entry into mitosis. As reflected by timing of mitotic entry and p53 serine15 phosphorylation, ATR cooperates with ATM in early responses to IR and is essential for late phase responses. While deletion of either gene alone does not completely eliminate the cell cycle delay induced by IR, deletion of both ATR and ATM causes an absence of any delay, either early or late, as compared with unirradiated cells. These results indicate that at least some functions of ATR and ATM are non-epistatic in the IR checkpoint response. Consistent with this interpretation, Chk2 phosphorylation in response to IR does not require ATR and is completely dependent on ATM. These results and others detailing the cooperative and singular roles of ATR and ATM in response to other forms of DNA damage will be discussed.

205. Development of mobile group II introns for mammalian gene targeting

Huatao Guo

Mobile group II introns can be designed for efficient gene targeting in bacteria. These introns are not only ribosomes, but also transposable elements. Intron transposition is mediated by the intron-encoded DNA endonuclease, which is a ribonucleoprotein (RNP) particle, containing both the intron RNA and the intron-encoded protein. The DNA target site for intron transposition is long (~31 nucleotides), therefore highly specific. The DNA target site is primarily recognized by the intron RNA through base pairing, allowing the intron to be flexibly retargeted to virtually any genes predictably.

To develop the Ll.LtrB intron for efficient mammalian gene targeting, we decided to directly express the Ll.LtrB RNP particles in mammalian cells. We have now achieved efficient expression of the intron-encoded protein in mammalian cells. Intron RNA can also be generated in mammalian cells. In addition, we have observed protein-dependent intron RNA splicing *in vivo*. We are now determining if the Ll.LtrB intron-encoded DNA endonuclease expressed in mammalian cells is able to cleave its DNA target sites. In addition, we will find out if the Ll.LtrB intron can insert into chromosomal DNA target sites in mammalian cells. If mobile group II introns can be developed to integrate into chromosomal DNA target sites efficiently, they could have broad applications in gene targeting and gene therapy.

206. The role of NF-κB in cellular transformation and cancer

Jeff Wiezorek, David Baltimore

The NF-κB transcription factor is composed of protein homo-and heterodimers of five gene products (p65, p50, p52, c-Rel, and RelB). Diverse stimuli act through the IκB kinases (IKKs) to promote the degradation of IκB and allow NF-κB translocation to the nucleus. NF-κB activation has been implicated in several biological processes including inflammation, immunoregulation, control of apoptosis, and cell proliferation. Although the transforming ability of the v-REL oncoprotein was established many years ago, recent evidence suggests other human NF-κB family members may be important in cellular transformation. NF-κB DNA binding activity is constitutively increased in many lymphoid and epithelial tumors. The RAS, BCR-ABL, and ERBB2 oncogenes can activate NF-κB. Furthermore, several genes thought to be essential to the cancer phenotype—those controlling angiogenesis, invasion, proliferation, and metastasis, contain κB binding sites. However, direct genetic evidence demonstrating the role of NF-κB in transformation and cancer progression is lacking.

Our lab has generated strains of knockout mice in which one or more of the NF-κB family members are deleted. 3T3 and primary fibroblasts of a specific genotype generated from these mice are powerful tools for dissecting NF-κB signaling pathways. These cells and mice are being used to study different aspects of cellular transformation and cancer progression.

An essential role for the NF-κB proteins in ERBB2 and RAS transformation through the activation of cyclin D1 is being investigated in primary and immortalized knockout fibroblasts. This signaling pathway may be important in some breast cancers. Another area of interest is the activation of NF-κB by tumor hypoxia. Hypoxic tumor cells are more resistant to therapy. NF-κB DNA binding increases in cells incubated under hypoxic conditions. The signaling pathways and physiologic consequences of this NF-κB activation are being studied in knockout fibroblasts. Finally, the role of NF-κB in the cancer phenotype is being evaluated *in vivo.* MMTV-RAS and transgenic mice readily develop mammary carcinomas at an early age. By crossing this transgene into NF-κB knockout strains, the contribution of constitutive NF-κB activity to tumor initiation, hypoxic survival, invasion/metastasis, and angiogenesis can be directly studied.

207. CARD11 mediates factor-specific activation of NF-κB by the T-cell receptor complex

Joel L. Pomerantz, Elissa M. Denny, David Baltimore

NF-κB is a transcription factor that functions as a pleiotropic regulator of genes involved in inflammation, the development and function of the immune system, and in antiapoptotic responses. NF-κB is commonly composed of two subunits, p50 and p65, which are held inactive in the cytoplasm by the binding of an inhibitor molecule, IκB. A variety of extracellular signals activate NF-κB by inducing the targeted destruction of IkB, thereby liberating NF-κB to translocate to the nucleus, bind to specific target sites, and activate a program of gene expression. Signals induce IκB degradation by activating the catalytic activity of a kinase complex (IKK complex) which phosphorylates IκB, targeting IκB for ubiquitination and destruction by the 26S proteasome.

Mice deficient in NF-κB subunits, or in molecules that signal to NF-κB, have revealed that the proper regulation of NF-κB activity is critical for normal innate and adaptive immune responses. In the adaptive immune response, NF-κB is a critical target of antigen receptor signaling in B and T cells. In T cells, the activation of NF-κB by T-cell receptor (TCR) triggering, in concert with costimulatory signals (CD28), is required for T-cell activation and proliferation. In addition, the properties of mice transgenic for nondegradable forms of IκB have suggested a role for NF-κB in TCR-mediated thymocyte selection and in pre-TCR survival signals.

The mechanisms by which TCR signaling activates the IKK complex are not well understood. Recently we have developed an expression cloning strategy for the isolation of components of signaling pathways that activate NF-κB. In our strategy, a cDNA expression library is assayed in pools for the ability to activate an NF-κB-responsive reporter when cotransfected into tissue culture cells. Positive pools are tested for specificity using a reporter containing mutated NF-κB binding sites. In addition, positive pools are tested in the presence of a kinase-dead IKKb subunit to confirm that they activate NF-κB through the activation of the IKK complex. Clones responsible for a particular pool's activity are purified by sib selection.

Using this strategy, we screened a mouse thymus expression library and cloned CARD11, a signaling adaptor molecule containing CARD, coiled-coil, PDZ, SH3, and GUK domains. Using dominant-negative and RNA-interference approaches, we demonstrated that CARD11 mediates the activation of NF-κB by TCR signaling. Importantly, we showed that the role of CARD11 is pathway-specific and factor-specific. CARD11 did not mediate NF-κB activation by TNFα or dsRNA, and CARD11 did not mediate the activation of the NFAT and AP-1 factors by TCR signaling. CARD11 functions upstream of the IKK complex and functionally cooperates with Bcl10, a signaling molecule overexpressed in mucosa-associated lymphoid tissue (MALT) lymphomas. Currently we are investigating how CARD11 functions mechanistically to transmit signals between the TCR and IKK complexes.

208. Specificity of transcriptional activation in the NF-κB/Rel protein family

Thomas Leung, David Baltimore

The study of mammalian gene transcription is often complicated by the fact that multiple members of a transcription factor family recognize the same regulatory sequence. While several models have been proposed, little is known about how genes recruit specific members of transcription factor families for activation. The Nuclear Factor kappa B (NF-κB)/Rel transcription factor family is an evolutionarily conserved gene regulation system involved in the coordination of an organism's response to infection, stress, and injury. Many different stimuli activate NF-κB, but the transcriptional response to each stimulus is unique. Within the NF-κB/Rel protein family, four members are involved in gene activation: p50, p52, p65, and cRel. They homo- or heterodimerize with one another to bind DNA.

Our lab has developed a genetic system to elucidate whether specific NF-kB/Rel family members are required for gene activation in a physiological manner. Single and multiple knock-outs for each member of the NF-κB/Rel family have been created, and 3T3 fibroblast cell lines have been derived. Our recent genetic analysis with this comprehensive panel of knock-out cell lines shows that NF-κB-dependent genes have specific NF-κB/Rel family member requirements for activation by TNFa. This finding suggests that the variety of NF-κB/Rel dimer isoforms allows for stimulus-specific gene expression programs.

How is the requirement for specific NF-κB/Rel family members generated? I am studying this question in three different ways. First, I will examine whether functional κB-binding-site sequences are strictly conserved in mammals. The recently completed genome sequences of mouse and human allow us to use evolution as a tool to help determine the importance of kB-binding-sites, and chromatin immunoprecipitation assays (ChIP) will be used to confirm our findings. Next, I plan to identify what promoter sequences are needed for the NF-κB/Rel family member requirement to be transferred from gene to gene. I will study promoter element requirements by using retroviral constructs that mediate chromosomal integration. Finally, I will determine if a promoter's requirement for specific NF-κB/Rel family members changes with different stimuli and elucidate a potential mechanism to account for stimulus-specificity.

209. Analysis of TNF and Fas receptor signaling to activation of gene expression

Mark Boldin, David Baltimore

Members of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily play a crucial role in activation, proliferation, survival and death of cells in the immune system. The two prototype death receptors of the family are type I TNF receptor (TNFR1) and Fas/APO-1. The biological functions triggered by these two receptors are characterized by a remarkable duality – infliction of tissue damage and cell death goes hand in hand with activation of tissue repair and expansion. The physiological reason for this duality lies in the ability of these receptors to trigger two kinds of intracellular signaling programs: a proteolytic cell death cascade and a number of kinase cascades leading to activation of gene expression, some of which may protect cells against cytotoxicity. The major transcriptional factors activated in response to TNF and Fas ligand are NF-κB and AP-1 proteins.

Despite significant recent progress in understanding the molecular mechanisms of TNFR1 and Fas receptor signaling towards activation of gene expression, there is still a great deal of confusion and argument with regard to the exact molecular scheme of events. The main cause for much of the disagreement in the field is the use of dominant-negative mutants and overexpression studies in order to elucidate the role of specific players in the pathway. Our rationale is that such approaches should be complemented by genetic studies. Genetic approaches often provide more clear-cut answers to the question of which molecules play an essential role in a given signal transduction pathway. Therefore, in order to identify molecules that play a crucial role in relaying the signals from the TNFR1 and Fas receptors towards NF-κB and AP-1 activation, we are performing genetic screens similar to that developed by George Stark and his colleagues for the analysis of interferon receptor signaling (1). The method is based on creation and subsequent genetic complementation of mutant mammalian cell lines that are defective in cytokine-induced activation of a signal transduction pathway leading to transcriptional activation.

Reference

(1) Pellegrini, S., John, J., Shearer, M., Kerr, I.M. and Stark, G.R. (1989) *Mol. Cell Biol.* **9**:4605-4612.

210. The regulation of gene targeting in vertebrate somatic cells

Matthew Porteus, David Baltimore

Gene targeting is a process by which exogenous DNA replaces endogenous genomic DNA. Such replacement can create subtle or dramatic sequence changes and is a tool that is now widely used in the study in yeast and mouse genetics. Theoretically, gene targeting is a powerful way to perform gene correction-type gene therapy. The low overall rate of gene targeting, in mammalian somatic cells, however, has precluded such use.

In order to understand gene targeting at a fundamental level, we developed flow cytometric assays based on the expression of GFP for gene targeting and random integration. Using these assays we have shown that the absolute rate of gene targeting in mammalian cells is $1x10^{-6}$ and confirmed that the rate can be stimulated over 1000-fold by the generation of a DNA double-stranded break (DSB) in the target gene. Furthermore, we have found that the rate of DSB-mediated gene targeting is directly and linearly dependent on the amount of homology between the substrate and target, the amount of substrate introduced into the cell and the frequency with which double-stranded breaks are generated. Finally, we have found that chicken lymphocyte cell line DT40, which has been reported to have a high rate of gene targeting, does not have a significantly increased rate of homologous recombination, but instead has a suppressed rate of random integration of exogenous DNA which results in a high relative, but not high absolute, rate of gene targeting.

In conclusion, we have found that gene targeting represents the final outcome of the repair of a DNA DSB and that the major factors in increasing the rate of gene targeting is the creation of a DSB in the target gene and the suppression of random integration. We are currently investigating ways to generate sequence-specific DSBs and to suppress random integration.

211. Mechanisms of signal transduction pathways in Abl/Arg

Wange Lu, David Baltimore

The aim of this project is to study the mechanism of signal transduction of Abl/Arg family protein kinase. In *Drosophila, abl* acts within developing neurons to regulate fasciculation and axonal outgrowth. In mammals, the *abl* gene family consists of Abl and Arg. Abl knockout mice display a variety of phenotypes with low penetrance. $Arg^{-/-}$ mice physically develop normally, but exhibit many subtle behavioral abnormalities. However, Abl and Arg double knockout mice are embryonic lethal due to a severe neurulation defect. The signaling mechanism of Abl/Arg remains unclear in either system.

To identify the potential signal transduction pathway, we decided to knockout Abl in *C. elegans*. The RNAi mutation of Abl leads to defects similar to Vab-1 and Vab-2, whose mammalian homologues are Eph receptor tyrosine kinases and their ligands, respectively. We then moved to mammalian cells to study a possible Eph/Abl/Arg connection because of the availability of various Abl/Arg-deficient cell lines.

Eph receptor tyrosine kinases belong to the biggest family of receptor tyrosine kinases. They play essential roles in neuron migration, axon guidance and synaptic plasticity. Arg and Eph were found to interact with each other at the overexpression level in HEK cells. Deletion analysis showed that the C-terminus of Arg, which is unique for Arg, is required for binding Eph. This is consistent with the fact that Arg binds to Eph strongly. Similar study has shown that SAM domain in Eph plays essential roles in binding Arg.

Comparison of Eph activity in wild-type and Abl/Arg double knockout cell lines further demonstrate that Abl/Arg are required for Eph activation. Furthermore, Arg alone can rescue the Eph activity in the Abl/Arg double knockout cell lines, which is consistent with the observation that Arg interacts and phosphorylates Eph specifically. In primary hippocampal/cortical neurons, Eph activity is significantly reduced in the Arg knockout mice, compared with activity in the wild-type neurons. Since Eph receptors have been shown to interact with NMDA receptors and involved in both excitatory synapse and spine formation, we have looked at the roles of Arg in these areas, especially with the knowledge that Arg knockout mice have some subtle behavior defects. NMDA receptor phosphorylation in both wild-type and knockout mice are at the same level. We are currently looking at both synapse and spine formation in Arg knockout and wild-type mice.

212. Molecular mechanism of $CD4^+CD25^+$ regulatory T cell-mediated immunosuppression

Xiao-Feng Qin, David Baltimore

Naturally occurring $CD25^+CD4^+$ T regulatory cells (Tr) represent a distinct sub-lineage of thymic dependent αβ T cells that can potently suppress immune responses of conventional $CD4^+$ and $CD8^+$ T cells (Tc) to self and innocuous foreign antigens. Although the physiological significance of this immunosuppressive function in peripheral tolerance and autoimmunity has been well appreciated, the molecular mechanism by which $CD25^+$ Tr cells suppress T-cell activation remains unknown. Our research in this area has focused on identifying cell surface molecules and signal transduction events involved in regulatory cell mediated contact-dependent suppression. We have recently found that the costimulatory receptor CTLA-4 plays an essential role in $CD25^+$ Tr suppressor function. In the absence of CTLA-4, $CD4^+$ T cells induce lethal autoimmune syndrome in Rag1 deficient host animals. However, the disease can be completely inhibited by cotransfer of wild-type $CD25^+$ Tr cells; whereas cotransfer of wild-type $CD25^-$ Tc cells fails to do so. Using bone marrow chimeras, we have further shown that CTLA-4 is not required for the development or peripheral maintenance of $CD25^+$ Tr cells but is essential for them to suppress autoreactive cells. While CTLA-4 is indispensable for $CD25^+$ Tr mediated autoimmune suppression *in vivo*, *in vitro* experiments using either polyclonal or antigen-specific models show CTLA-4 mutant $CD25^+$ Tr cells still possess weak suppressor activity, particularly at high $CD25^+$ Tr to $CD25^-$ Tc ratios. Therefore, these results suggest there might be other pathways utilized by $CD25^+$ Tr cells to suppress T-cell activation under different circumstances. Alternatively, CTLA-4 might just be part of a multi-subunit signaling complex, and removal of CTLA-4 does not completely disable its function. To address this and other issues, we plan to use a signal-peptide-trap cloning approach to clone Tr-specific surface molecule genes. In addition, we have also recently developed a high efficient way to knock down gene expression using lentiviral-mediated delivery of siRNA driven by pol III promotes. By combining these two technologies together, we will be able to systematically identify candidate genes and test their function in regulatory mediated immunosuppression in various settings.

213. Function of neuronal NF-κB

Mollie K. Meffert, David Baltimore

Long-lasting alterations of neuronal properties, such as those involved in plasticity and apoptosis, depend upon changes in gene expression. We are exploring the neuronal function of a potent transcription factor, Nuclear Factor kappa B (NF-κB) in mice. NF-κB is the prototype of a family of dimeric transcription factors made from monomers that have Rel regions that bind DNA, interact with each other, and also bind the IκB inhibitors. Although NF-κB has been most thoroughly studied in cells of the immune system, a role for NF-kB in neuronal (and glial) cell function has recently been proposed.

In mature unstimulated hippocampal neurons, a low level of basal κB DNA-binding activity is present. We have found that the NF-κB transcription factor is activated by basal synaptic input in a calcium-dependent-pathway requiring activity of the calcium-calmodulin dependent kinase, CaMKII. In addition, both physiological as well as pathological stimuli can further induce κB-binding activity consisting of p50:p65 and p50:p50 dimers (measured by EMSA). Inducible NF-κB resides in the cytoplasm complexed to IκB; cytoplasmic storage enables it to receive upstream signals more directly than transcription factors localized solely to the nucleus and also could allow it to transport signals from distant synaptic stimuli to the nucleus. We constructed a GFP-p65 fusion gene to explore NF-κB localization and translocation in neurons. Our GFP-p65 fusion protein localizes to both the cell body and processes of neurons and undergoes nuclear translocation following stimulation by glutamate or depolarization. Experiments using synaptosomes (isolated nerve terminals) indicates that both p50 and p65, as well as IκBα and IκBβ are found at synapses. While both p50:50 and p50:p65 dimers occur in intact neurons, synapses contain only p65:p50. We have confirmed this interesting finding using a p65 knockout mouse made in our laboratory. While intact neurons from this p65 null mouse contain p50:p50 dimers, no NF-κB or IkB can be detected at synapses. The p65 knockout mouse thus provides an effective system to address the specific functions of synaptic NF-κB. Ribonuclease protection experiments using cultured p65-deficient neurons have revealed that NF-κB regulates the transcription of diverse neuronal genes, some that modulate synaptic function and others that are markers of synaptic activity. Furthermore, mice lacking p65 exhibit a learning deficit in a spatial version of the radial arm maze task.

214. Generation of functional antigen-specific T cells in defined genetic backgrounds by retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells

Lili Yang

We have developed an alternative to transgenesis for producing antigen-specific T cells *in vivo*. In this system, clonal naïve T cells with defined antigen specificity are generated by retrovirus-mediated expression of TCR cDNAs in RAG1-deficient murine hematopoietic precursor cells. These T cells can be stimulated to proliferate and produce cytokines by exposure to antigen *in vitro*, and they become activated and expand *in vivo* following immunization. IL-2-deficient T cells generated by this technique show decreased proliferation and cytokine production, both of which can be rescued by exogenous addition of this growth factor. Thus, retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells permits the rapid and efficient analysis of the life history of antigen-specific T cells in different genetic backgrounds, and may allow for the long-term production of antigen-specific T cells with different functional properties for prophylactic and therapeutic purposes.

Publications

Dadgostar, H., Zarnegar, B., Hoffmann, A., Qin, X-F., Truong, U., Rao, G., Baltimore, D. and Cheng, G. (2002) Cooperation of multiple signaling pathways in CD40-regulated gene expression in B lymphocytes. *Proc. Natl. Acad. Sci. USA* **99**:1497-1502.

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**:868-872.

Yang, L., Qin, X-F., Baltimore, D. and van Parijs, L. (2002) Generation of functional antigen-specific T cells in defined genetic backgrounds by retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells. *Proc. Natl. Acad. Sci. USA* **99**:6204-6209.

Professor of Biology: Pamela J. Bjorkman
Research Fellows: Melanie Bennett, Mindy Davis, Andrew Herr, W. Lance Martin, Christopher O'Callaghan, T.S. Ramalingam, Elizabeth R. Sprague, Anthony West, Benjamin E. Willcox
Graduate Students: Tony Giannetti, Agi Hamburger, Zsuzsa Hamburger, Devin Tesar
High School Students: Ed Ballister, Elizabeth Leyton, Adam Paiz
Research and Laboratory Staff: Silvia Delker, Beth Huey-Tubman, Lynda Llamas, Marta Murphy, Leonard Thomas, Noreen Tiangco

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Summary: My laboratory is interested in the structure and function of molecules involved in cell surface recognition, particularly those mediating recognition in the immune system (**Figure 1**). We use a combined approach of x-ray crystallography to determine structures, molecular biological techniques to produce proteins for crystallization and to modify them, and biochemistry to study the properties of the proteins we make. Much of our work has focused upon homologs of class I MHC proteins, which function in many ways that are distinct from their immunological role in peptide presentation to T cells. Our interest in these types of proteins has grown since we solved the structure of the neonatal Fc receptor (FcRn), an MHC homolog that transports immunoglobulin G (IgG). In the FcRn system, we are using structural information to address cell biological issues involving intracellular receptor-ligand trafficking. Our studies of other MHC homologs have expanded our interests to include aspects of iron metabolism (for our work on HFE), cancer (for our work on Zn-α2-glycoprotein; ZAG), and virology (for our work on viral MHC homologs).

Transfer of maternal IgG molecules to the fetus or infant is a mechanism by which mammalian neonates acquire humoral immunity to antigens encountered by the mother. The protein responsible for the transfer of IgG is the MHC class I-related receptor FcRn. MHC class I molecules have no reported function as immunoglobulin receptors; instead they bind and present short peptides to T cells as part of immune surveillance to detect intracellular pathogens. We solved the crystal structures of rat FcRn both alone and complexed with Fc. We have hypothesized that formation of an oligomeric ribbon of FcRn dimers on adjacent membranes bridged by IgG molecules (**Figure 2**) is required for FcRn function. We are now interested in understanding the roles of the FcRn dimer and the oligomeric ribbon in IgG transport. We are in the process of characterizing oligomeric ribbon formation *in vitro* using biophysical assays, and have extended these studies to real-time confocal imaging of cells undergoing FcRn-mediated transcytosis of IgG. We are also beginning structure/function studies of two other Fc receptors that are not MHC homologs: gE/gI, a viral Fc receptor for IgG, and FcαR, a host receptor for IgA.

HFE is a recently discovered class I MHC homolog that is involved in the regulation of iron metabolism, an unexpected function for an MHC-related protein. HFE was discovered when its gene was found to be mutated in patients with the iron overload disease hereditary hemochromatosis. HFE has been linked to iron metabolism with the demonstration that it binds to transferrin receptor, the receptor by which cells acquire iron-loaded transferrin. We solved crystal structures of HFE alone and HFE bound to transferrin receptor. The interaction of HFE with transferrin receptor is a fascinating system to study because we can use crystal structures to answer biochemical, functional, and evolutionary questions that address how binding of HFE interferes with transferrin binding, if conformational changes in the receptor are involved in the binding of either transferrin or HFE, which part of the MHC-like HFE structure binds transferrin receptor, and how the HFE interaction with the receptor compares with interactions of ligands with MHC and MHC-like (e.g., FcRn) proteins. We are expanding our studies to include cell biological investigations of HFE and transferrin receptor intracellular trafficking in transfected cell lines using confocal microscopy and other imaging techniques.

We also study Zn-α2-glycoprotein (ZAG), a soluble MHC class I homolog present in low concentrations in most bodily fluids. ZAG was isolated from blood more than 30 years ago, but it's been a molecule in search of a function for a long time. Recently researchers at Aston University in the U.K. discovered that ZAG is involved in cachexia, a wasting syndrome that can affect people with terminal illnesses. ZAG is responsible for the fat-depletion component of cachexia, since it stimulates lipid breakdown in adipocytes and reduces fat stores in laboratory animals. We have purified ZAG from serum and completed its crystal structure, which revealed an as yet unidentified non-peptide compound in the ZAG counterpart of the MHC peptide binding site. A combination of structural studies, computational analysis, and ligand binding experiments will be used for analyzing the function and potential roles of ZAG in lipid catabolism under normal and pathological conditions.

We are also interested in other MHC homologs, including proteins encoded by viruses. Both human and murine cytomegalovirus (HCMV, MCMV) express a relative of MHC class I heavy chains, probably as part of the viral defense mechanism against the mammalian immune system. Our biochemical studies show that the HCMV homolog associates with endogenous peptides resembling those that bind to class I MHC molecules. Current efforts focus upon defining the structure and

function of these homologs in order to understand why viruses make them and how they interfere with the host immune system.

Our structural work on class I MHC homologs has elucidated new and unexpected recognition properties of the MHC fold. For FcRn and HFE, the structural and biochemical studies have revealed a similar fold and some common properties, including the assumption that both receptors "lie down" parallel to the membrane when binding ligand, and a sharp pH-dependent affinity transition near neutral pH. In the case of FcRn, we have elucidated the structural basis of its pH-dependent interaction with IgG and will now focus upon cell biological studies of intracellular trafficking, for which the pH-dependent interaction is critical. The pH dependence of the HFE-TfR interaction suggested to us that intracellular trafficking studies of HFE would be interesting, so much of our future efforts on both the FcRn and HFE systems will center around probing their function in a cellular context using imaging techniques. Our functional studies of ZAG are at an earlier stage, since a receptor for ZAG has not been identified and the mechanism by which ZAG promotes lipid degradation is unknown. We are at an even earlier stage in our studies of the viral MHC homologs, in which our primary goal will be to solve crystal structures of one or both viral homologs alone and complexed with their cellular receptors.

In addition to the studies of MHC homologs, we are interested in using structure/function studies to understand bacterial pathogenesis and the innate immune response to bacterial infection. Our efforts in these areas involve the study of the *Yersinia pseudotuberculosis* protein invasin, the insect immune response protein hemolin, and the mammalian mannose receptor.

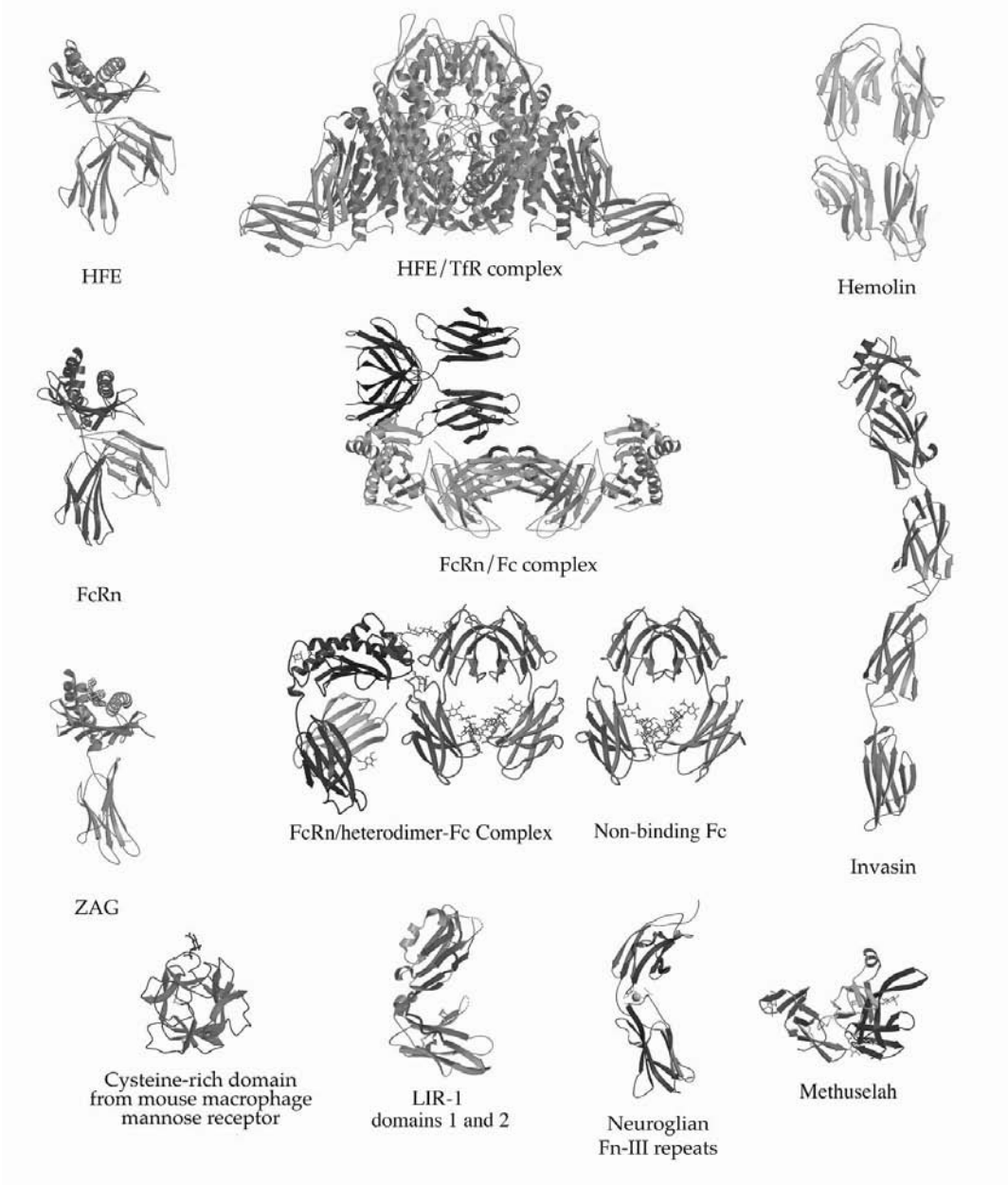

Figure 1.

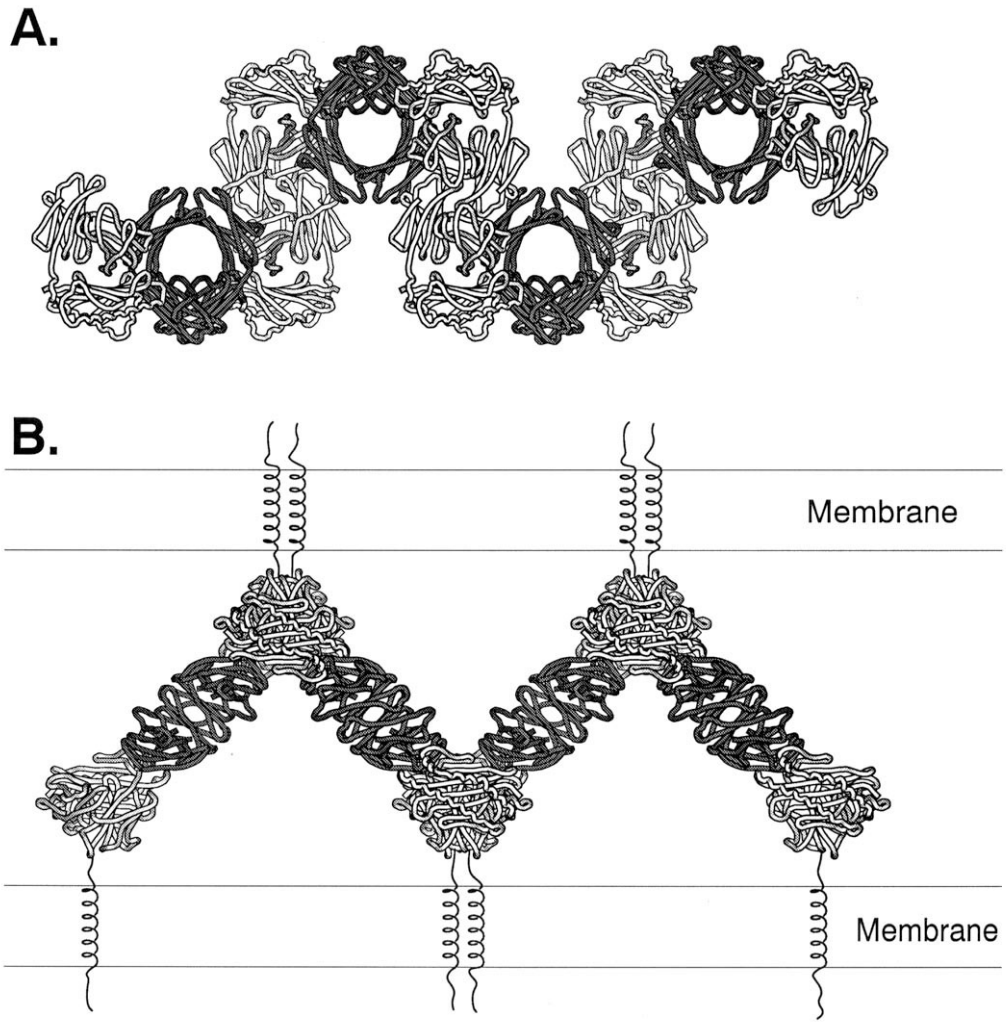

Figure 2.

215. Assay for ligand-induced dimerization of FcRn

*Devin B. Tesar, Mary Dickinson**

The 6.0 A crystal structure of FcRn complexed with wild-type Fc demonstrates the formation of a continuous array of FcRn dimers bridged by Fc ligands. This "oligomeric ribbon" may have functional implications for FcRn-mediated IgG transport in living cells. For example, transport vesicles containing the oligomeric ribbon would have the opposing membrane faces brought into close (~200 Å) proximity of one another. In addition, oligomeric ribbon formation would induce an ordered linear arrangement of FcRn cytoplasmic tails. The cytosolic trafficking machinery may recognize one or both of these features as a means to distinguish between vesicles that contain FcRn-IgG complexes and those that contain FcRn alone or unbound IgG. Oligomeric ribbon formation would require the simultaneous occurrence of two distinct phenomenon; 1) FcRn dimerization, and 2) the bridging of FcRn molecules on opposing membranes by Fc ligands. To test for ligand-induced FcRn dimerization under physiological conditions we have devised an *in vivo* assay using Fluorescence Resonance Energy Transfer (FRET). We have created chimeric FcRn molecules that either enhanced cyan fluorescent protein (ECFP) or enhanced yellow fluorescent protein (EYFP) fused the C-terminus of the cytoplasmic tail. In collaboration with Dr. Mary Dickinson in Scott Fraser's laboratory at Caltech, we will use laser scanning confocal microscopy in conjunction with a Zeiss META system, capable of deconvoluting the CFP and YFP spectra, to detect energy transfer from FcRn-ECFP to FcRn-EYFP in stably transfected Madin-Darby Canine Kidney (MDCK) cells, both in the presence and absence of IgG. These results will be compared to FRET measurements of engineered FcRn-CFP/YFP molecules containing putative dimerization-disrupting mutations. We will also test for FRET in HLA-DR, an MHC class II molecule and an obligate heterodimer, as a positive control and, as a negative control, between FcRn-ECFP and HFE-EYFP, an MHC class I homolog that does not bind IgG or interact with FcRn.

**Fraser Lab*

216. Cloning of the chicken yolk sac IgY receptor

Anthony P. West, Jr.

Like mammals, birds transmit antibodies to their young. Antibodies (IgY) are packaged into the yolk during egg formation and are selectively transmitted to the embryonic circulation across the yolk sac membrane. Previous studies have observed a membrane-bound receptor that binds IgY at pH 6 but not at neutral pH. This resembles the behavior of the mammalian counterpart, FcRn. We have purified a 150 kDa protein from detergent-solubilized chicken yolk sac membranes using pH-dependent binding to an IgY-affinity column. Using information from mass spec, protein microsequencing, and chicken ESTs, we were able to clone the cDNA for this protein by RACE-PCR. The chicken protein is not related to FcRn. Expression of this protein in transected CHO cells results in pH-dependent binding of labeled chicken Fc fragments. Further structural and functional studies will be conducted to compare the properties of this new Ig receptor to those of FcRn, its mammalian counterpart.

217. Stoichiometry and binding mechanism for the interaction between FcαRI and IgA

Andrew B. Herr, Clinton L. White, Christina Milburn**, Carol Wu****

FcαRI, the receptor specific for the Fc region of IgA, is responsible for IgA-mediated phagocytosis, oxidative burst, and antibody-dependent cellular cytotoxicity. We have shown that two FcαRI molecules bind to a single Fcα homodimer. Surface plasmon resonance studies confirm the 2:1 stoichiometry of binding, with similar affinities at each site. The binding affinity is pH-dependent, in a manner consistent with protonation of a histidine residue in the binding site. A thermodynamic analysis indicates that the histidine does not participate in a salt bridge in the complex; indeed, less than 7% of the free energy of binding was contributed by electrostatic interactions. The bivalent, pH-dependent interaction between FcαRI and IgA has important implications for cytokine-dependent phagocytosis of IgA and the FcαRI-mediated degradation or recycling of IgA.

**Campbell Alliance, Raleigh, NC*
***Medarex, Inc., Milpitas, CA*
****Center for Health Sciences, UCLA, Los Angeles, CA*

218. Structural studies of a herpes simplex virus immunoglobulin G receptor

Elizabeth R. Sprague, Elizabeth E. Leyton, Adam A. Paiz**

The herpes simplex virus (HSV) immunoglobulin G (IgG) receptor is a heterodimer composed of the gE and gI proteins that is found on the surface of the HSV virion or HSV-infected cells. The gE/gI heterodimer binds the Fc portion of IgG, and this interaction has been implicated in several mechanisms of immune evasion, including inhibition of virus neutralization, interference with complement- and cell-mediated lysis pathways, and antibody-bipolar bridging. The interplay between HSV proteins and host immunoregulatory proteins may contribute to the ability of HSV to establish a persistent infection even in the presence of anti-HSV antibodies. The gE protein has also been implicated in the cell-to-cell transmission of HSV. In order to facilitate understanding how the gE/gI heterodimer binds the Fc portion of IgG and how this recognition event affects the ability of the host immune system to eradicate the virus, we have undertaken biochemical and crystallographic studies of the gE protein, gE/gI heterodimer, and the gE/gI-Fc complex. Using baculovirus-assisted expression in insect cells, we have produced sufficient quantities of various pieces of the gE and gI proteins, both as separate proteins and as heterodimeric complexes. The crystal structure of one fragment of gE has been solved, and we are currently working on crystallizing other fragments. The Fc portion of human IgG1 is expressed in CHO cells. The gE/gI heterodimer with and without human Fc and each of the gE fragments have been extensively screened for crystallization under numerous conditions. Crystals of a gE/gI heterodimer in complex with human Fc show weak, anisotropic diffraction with reflections extending to 5.5 Å in the best and 7 Å in the worst direction. Since these ternary complex crystals are insufficient to acquire detailed structural information, we are currently screening for additional crystal forms and investigating additional gE/gI heterodimer and human Fc constructs. We are also building on the previous characterization of the interaction between human Fc and the gE/gI heterodimer (1), and have recently demonstrated that gE/gI shows strongly pH-dependent ligand binding properties, analogous to FcRn, but in the opposite direction (gE/gI binds tightly to IgG at neutral or basic pH, but not at acidic pH).

**High school student*

Reference

(1) Chapman, T.L., Heikema, A.P., West, A.P. and Bjorkman, P.J. (2000) *Immunity* **13**:727-736.

219. Structural studies of a retroviral envelope glycoprotein

*Agi Hamburger, Peter Kim**

One of the key steps in the retroviral life cycle is the delivery of the viral genome into the host cell cytoplasm. Viral entry is mediated by the interaction between the envelope glycoprotein complex (SU/TM) and the cellular receptor, whereby binding of the surface subunit (SU) triggers conformational changes in the transmembrane subunit (TM) and leads to the fusion of the two membranes. Although a number of crystal structures have been solved of the fusogenic state of the viral TM subunit, including Moloney Murine Leukemia Virus (Mo-MLV), HTLV-1, HIV and SIV, only a very limited amount of structural information is available about the native state of the envelope protein. Structural studies have been hindered in part because envelope glycoprotein complexes are difficult to produce in high quantities and the SU/TM complexes are unstable and easily dissociate. We have expressed the extracellular domain of the Mo-MLV in several expression systems, including bacteria, insect cells and mammalian cells. We are currently using biophysical and biochemical techniques to characterize the Mo-MLV ectodomain. Crystallization trials of the Mo-MLV ectodomain are also underway.

**Merck Research Laboratories, West Point, PA*

220. Characterization of protein ligand binding by the transferrin receptor

Anthony M. Giannetti

Transferrin receptor (TfR) is a homodimeric transmembrane protein. TfR binds iron-loaded transferrin (Fe-Tf), apo-Tf, and the Hereditary Hemochromatosis protein HFE. Ligand binding to TfR exhibits pH dependency and may involve structural rearrangements to the TfR dimer. TfR binds HFE at the basic pH of the cell surface (pH~7.4) but not at the acidic pH of endosomes (pH~6.0). Fe-Tf binds TfR at acidic and basic pH, but apo-Tf only binds at low pH. In a previous mutagenesis study of TfR[1] we demonstrated that HFE and Fe-Tf share an overlapping binding site and identified mutations that eliminate HFE binding without strongly reducing Fe-Tf binding. I have continued this study and identified additional mutations that affect Fe-Tf binding without strongly affecting HFE binding. The observation that HFE and Fe-Tf compete for binding predicts that at the cell surface HFE acts as a competitive inhibitor for Fe-Tf binding. I have tested this competition using human epithelial cells transfected with an HFE-GFP fusion construct and shown by confocal microscopy and flow cytometry that, at the cell surface, HFE competes for TfR binding quite well despite the high concentrations of Fe-Tf in serum. We would like to crystallize the ternary complex of HFE, TfR, and Fe-Tf. Fe-Tf has a much stronger binding affinity to TfR than does HFE, thus it is not possible to crystallize the ternary complex directly as the Fe-Tf will compete away the HFE to yield only the Fe-Tf/TfR complex. Therefore I have constructed a heterodimeric TfR in which one monomer of the dimer is competent to bind HFE but not Fe-Tf while the other monomer is able to bind Fe-Tf, but not HFE. This complex has been expressed and purified making use of different affinity tags on each monomer of the heterodimer.

Reference

West, A.P. Jr., Giannetti, A.M, Herr, A.B., Bennett, M.J., Nangiana, J.S., Pierce, J.R., Snow, P.M. and Bjorkman, P.J. (2001) *J. Mol. Biol*. **313**:385-397.

221. Structural and biophysical studies of transferrin receptor 2

*Mindy I. Davis, Caroline A. Enns**

The transferrin receptor 2 (TfR2), a type I membrane glycoprotein, binds iron-loaded transferrin (Fe-Tf), a protein critical to iron transport and metabolism. Previous surface plasmon resonance (SPR) results demonstrated that a homologous protein, transferrin receptor 1 (TfR1, 45% amino acid identity in the human extracellular regions), binds both Tf and HFE (1). This complex is then endocytosed, where at the lower pH of the endosome, iron is released from Fe-Tf. The iron is then transported to the iron storage protein ferritin and the apoTf/HFE/TfR1 complex is recycled to the cell surface. Unlike TfR1, TfR2 appears to bind only Tf and not HFE, as shown by SPR (2). The exact role in iron metabolism of TfR2 in comparison to TfR1 is not currently well understood although it is known that expression of TfR2 in CHO cells lacking TfR1 does allow for cell growth (3). A defect in either TfR2 or HFE leads to hereditary hemochromatosis, a disease of iron overload, and thus understanding the role of TfR2 in iron homeostasis is important. We are currently undergoing crystallization trials of the extracellular domain of TfR2, expressed by Peter Snow (Caltech Protein Expression Center) as a soluble protein in a baculovirus expression system. Additionally we are extending the previous SPR results to include the effect of pH on the binding of Tf, apoTf and HFE to TfR2. These results are anticipated to lead to an understanding of the different binding properties of TfR1 and TfR2 as well as shed light into the origins of hereditary hemochromatosis arising from mutations in TfR2.

**Oregon Health Sciences University, Portland, OR*

References

(1) Lebrón, J.A., West, A.P. and Bjorkman, P.J. (1999) *J. Mol. Biol*. **294**:239-245.
(2) West, A.P., Bennett, M.J., Sellers, V.M., Andrews, N.C., Enns, C.A. and Bjorkman P.J. (2000) *J. Biol. Chem*. **275**:28135-28138.
(3) Kawabata, H., Germain, R.S., Vuong, P.T., Nakamaki, T., Said, J.W. and Koekkler, H.P. (2000) *J. Biol. Chem.* **275**:16618-16625.

222. Crystal structure of LIR-2: Implications for targeting by an HCMV class I MHC homolog

Benjamin E. Willcox, Leonard Thomas, Tara Chapman, Astrid Heikema**, Anthony P. West*

Leukocyte Immunoglobulin-like Receptor-1 (LIR-1) and LIR-2 are highly related cell surface receptors which bind a broad range of class I MHC molecules with low (μM) affinities. Expressed on monocytes and macrophages, both molecules transmit inhibitory signals after binding ligand (1). In addition to binding host class I MHC, the LIR-1 molecule, which is also expressed on lymphoid tissues, binds with high (nM) affinity to UL18, a class I MHC homolog encoded by Human Cytomegalovirus (HCMV) (2). In comparison, LIR-2 binds UL18 only weakly (Kd μM) (3). The structure of LIR-1 was recently solved (3). To understand how HCMV preferentially targets the more broadly expressed LIR-1 molecule, we determined the structure of LIR-2 to 1.8 Å and identified conformational differences between the two receptors that might explain differential binding to UL18. The overall structure of LIR-2 D1D2 resembles both LIR-1, and Killer Inhibitory Receptors (KIRs). However, differences to LIR-1 are observed in each domain, with two key changes apparent in the ligand-binding domain, D1. The region corresponding to the α-helix of LIR-1 (residues 50-65) adopts a topology distinct from that of both LIR-1 and the KIR structures, involving a shortened helix. Secondly, the region of LIR-1 predicted on the basis of site-directed mutagenesis data to form the binding site for UL18 (3) is altered substantially in LIR-2. In summary the structure of LIR-2 has revealed significant differences to LIR-1, including the ones that may help to explain the 1000-fold lower affinity of LIR-2 for UL18.

**Amgen, Thousand Oaks, CA*
***Erasmus MC, Medical Microbiology and Infectious Diseases, The Netherlands*

References

(1) Borges, L. and Cosman, D. (2000) *Cytokine Growth Factor Rev.* **11**:209-217.

(2) Cosman, D., Fanger, N., Borges, L., Kubin, M., Chin, W., Peterson, L. and Hsu, M.L. (1997) *Immunity* **7**:273-282.

(3) Chapman, T.L., Heikema, A.P., West, A.P., Jr. and Bjorkman, P.J. (2000) *Immunity* **13**:727-736.

223. Characterization and crystallographic studies of the mouse and rat cytomegalovirus-encoded major histocompatibility complex class I homologs, m144 and r144

Astrid Heikema, Kathryn E. Huey-Tubman*

Cytomegaloviruses (CMVs) have evolved strategies to modulate the host immune response that allow the establishment of persistent infection. Human (HCMV), murine (MCMV) and rat (RCMV) CMVs encode homologs of major histocompatibility complex (MHC) class I molecules. MHC class I molecules present endogenous peptides, thus enabling cytotoxic T cells to detect and destroy virus-infected cells. CMV-infected cells down-regulate the expression of host MHC class I molecules and thereby evade eradication by cytotoxic T cells. However, down-regulation of MHC class I molecules on the surface of infected cells lends them susceptible to Natural Killer (NK) cell-mediated clearance. Therefore, it has been hypothesized that expression in virus-infected cells of the MHC class I homologs, UL18 (HCMV), m144 (MCMV) and r144 (RCMV) prevent the lysis that would normally occur by cytotoxic T cells due to down-regulation of MHC class I molecules, and allow viral persistence.

The MHC class I homologs UL18, m144 and r144, like MHC class I molecules, consist of a heavy chain containing three extracellular domains (α1, (α2, and (α3) that associates with the host β2-microglobulin (β2m) light chain. Previous studies in our lab have shown that like MHC class I molecules, UL18 binds endogenous peptides, whereas m144 and r144 do not. The peptide binding site in MHC class I molecules is located in a groove that is formed by two α-helices in the α2 domain. Alignments of the predicted amino acid sequence of m144 and r144 with UL18 show a significant truncation in the α2 domain of both m144 and r144, which may explain the non-peptide-binding nature of m144 and r144. To investigate this further by crystallographic studies, r144 has been expressed in *E. coli*, purified from inclusion bodies, refolded with bacterial-expressed rat β2m and purified using size exclusion chromatography. The purified protein has been used to initiate crystallization trials. Bacterial expression constructs for m144 and mouse β2m are in progress and resulting purified protein will also be used for crystallization trials.

**Erasmus MC, Medical Microbiology and Infectious Diseases, The Netherlands*

224. Bacterial expression of Zn-α_2-glycoprotein: Investigating the structural and functional importance of ligand binding within the groove

*Anthony P. West, Jr., Silvia L. Delker, Astrid Heikema**

Zn-α_2-glycoprotein (ZAG) is a 41 kDa soluble protein that is present in most bodily fluids. Recent studies by Michael Tisdale and his colleagues at Aston University have suggested that ZAG plays a role in the extreme weight loss (cachexia) that occurs in some cancer patients (1). Since 30-50% of cancer mortality is a direct result of this wasting syndrome, developing therapies to reduce the systemic strain associated with wasting is an important goal. We previously determined the 2.8 Å crystal structure of ZAG isolated from human serum demonstrating the structural similarity between ZAG and MHC I molecules (2). Unlike MHC I molecules, ZAG was shown to bind a small non-peptidic ligand within its groove. The importance of this ligand in ZAG's structure and function is unclear. If a ZAG molecule devoid of this ligand could be produced, the ligand's role in lipid breakdown could be evaluated. We have expressed human and mouse ZAG in *E. coli* and successfully refolded these proteins. Since bacteria and mammals utilize vastly different lipid metabolism pathways, the expectation is that these forms of ZAG will have empty binding grooves. We have obtained crystals of refolded human ZAG which diffract to 4.2 Å and are in the process of optimizing them in order to proceed with a crystallographic structure determination.

**Erasmus MC, Medical Microbiology and Infectious Diseases, The Netherlands*

References

(1) Todorov, P.T., McDevitt, T.M., Meyer, D.J., Ueyama, H., Ohkubo, I. and Tisdale, M.J. (1998) *Cancer Res.* **58**:2353.

(2) Sanchez, L.M., Chirino, A.J. and Bjorkman, P.J. (1999) *Science* **283**:1914.

225. Structural studies of invasin

Zsuzsa A. Hamburger, Kaspar P. Locher, Adam T. Politzer**, and Ralph R. Isberg****

Bacterial pathogens, such as *Yersinia pseudotuberculosis*, must bind and enter normally non-phagocytic cells to establish infection. The protein responsible for mediating uptake of the bacterium is a 986-residue outer membrane protein called invasin. Invasin binds to several members of the β_1 integrin family, presumably activating a reorganization of the host cytoskeleton to form pseudopods that envelop the bacterium. Integrin binding has been localized to the extracellular region of invasin (Inv497) comprised by the COOH-terminal 497 residues. In order to gain insight into host cell entry by *Yersinia pseudotuberculosis*, we solved the 2.3 Å crystal structure of Inv497 (1). The structure reveals five domains that form a 180 Å rod with structural similarities to tandem fibronectin-III domains. The integrin-binding surfaces of invasin and fibronectin include similarly located key residues, but in the context of different folds and surface shapes. The structures of

invasin and fibronectin provide an example of convergent evolution, in which invasin presents an optimized surface for integrin binding compared with host substrates. We have also initiated structural analyses of the NH_2-terminal ~500 residues of invasin, which are required for outer membrane localization and for presentation of the integrin-binding region of invasin. We expressed this region of invasin as inclusion bodies in *E. coli*, and refolded the protein in the presence of detergents. We have also obtained microcrystals of this membrane protein. Circular dichroism studies indicate that this region of invasin is composed of mainly β-structure. As the transmembrane regions of outer membrane proteins of known structure are β-barrels, this region of invasin is also presumed to fold into such a structure. Proteolysis experiments suggest that the N-terminal 70 amino acids of invasin may form a separate domain from the invasin transmembrane region, analogous to that found in another outer membrane protein, the sucrose-specific porin ScrY. Equilibrium sedimentation analytical ultracentrifugation studies indicate the protein is monomeric in solution. Black lipid bilayer experiments suggest that this region of the protein does not contain a pore and thus plays the role of an outer membrane anchor for the presentation of the integrin-binding domain on the cell surface.

*Postdoctoral fellow (Rees lab), Division of Chemistry and Chemical Engineering
**High school student, Flintridge Preparatory School
***HHMI, Tufts University

Reference

(1) Hamburger *et al*. (1999) *Science* **286**:291-295.

226. A linear lattice model for poly-glutamine in CAG expansion diseases

*Melanie J. Bennett, Kathryn E. Huey-Tubman, Andrew B. Herr, Anthony P. West, Jr., Scott A. Ross**

Huntington's Disease and several other neurological diseases are caused by expanded poly-glutamine (poly-Gln) tracts in different proteins. Mechanisms for expanded poly-Gln (>36 Gln) toxicity include the formation of aggregates that recruit and sequester essential cellular proteins (1,2) or functional alterations, such as improper interactions with other protein (3). Expansion above the "pathologic threshold" (~36 Gln) has been proposed to induce a conformational transition in poly-Gln tracts, which has been suggested as a target for therapeutic intervention. Our structural analyses of soluble huntingtin Exon 1 fusion proteins with 16 to 46 glutamines reveal extended structures with random coil characteristics and no evidence for a global conformational change above 36 glutamines. An antibody (MW1; produced in Paul Patterson's laboratory) Fab fragment, which recognizes full-length huntingtin in mouse brain sections, binds specifically to Exon 1 constructs containing normal and expanded poly-Gln tracts, with affinity and stoichiometry that increase with poly-Gln length. These data support a "linear lattice" model for poly-Gln, in which expanded poly-Gln tracts have an increased number of ligand binding sites as compared to normal poly-Gln. The linear lattice model provides a rationale for pathogenicity of expanded poly-Gln tracts and a new structural framework for drug design.

*Mayo lab, Caltech

References

1. Preisinger, E., Jordan, B.M., Kazantsev, A. and Housman, D. (1999) *Phil. Trans. R. Soc. Lond. B Biol. Sci.* **354:**1029-1034.
2. Chen, S., Berthelier, V., Yang, W. and Wetzel, R. (2001) *J. Mol. Biol.* **311**:173-182.
3. Cummings, C.J. and Zoghbi, H.Y. (2000) *Hum. Mol. Genet.* **9**:909-916.

227. In search of the ligand of Methuselah, a *Drosophila* GPCR associated with extended lifespan

Anthony P. West, Jr., Silvia L. Delker, Laurent Seroude, Seymour Benzer**

The *Drosophila* mutant *methuselah* was identified from a screen for single-gene mutations that extended average life span. The *methuselah* mutant has a 35% increase in average life span and increased resistance to several forms of stress. The protein affected by this mutation appears to be a G protein-coupled receptor (GPCR), though the upstream and downstream pathways that Methuselah (Mth) connects are unknown. Mth has a large extracellular N-terminal domain, which may be the ligand-binding domain of the receptor by analogy to related GPCRs, whose isolated extracellular domains function in ligand binding. We have determined the 2.3 Å crystal structure of the Mth ectodomain. The structure did not closely resemble any other protein and thus did not suggest what the natural ligand might be. Nevertheless, the ectodomain structure reveals a potential ligand-binding site, in a shallow groove between two flexible domains.

We are attempting to identify the natural ligand and G-protein effector of Mth, which should help us to understand how Mth affects *Drosophila* lifespan. The basic plan is a biochemical approach: *Drosophila* extracts will be applied to cells expressing full-length Mth, the system will be varied until Mth activation is observed, and the extract will be fractionated until the ligand can be identified. We are exploring two assay systems that have been used to identify the ligands of other orphan GPCRs: (i) *Xenopus* oocytes injected with receptor mRNA, with electrophysiological monitoring of receptor activation; and (ii) transfected mammalian cells, where GPCR activation can be followed by increases in intracellular calcium. Coexpression of Mth with a 'universal G protein' provides a method to follow Mth activation without assumptions concerning its normal signaling pathway. We have raised monoclonal antibodies against the Mth ectodomain for immunoprecipitation of Mth from *Drosophila* membranes. G proteins, or other interacting proteins, may coimmunoprecipitate with Mth, and identifying these proteins should assist in developing the Mth assay.

*Benzer lab

Publications

Bennett, M.J., Huey-Tubman, K.E., Herr, A.B., West, A.P., Jr., Ross, S.A. and Bjorkman, P.J. (2002) A linear lattice model for polyglutamine in CAG expansion diseases. *Proc. Natl. Acad. Sci. USA* **99**:11634-11639.

Bennett, M.J., Lebrón, J.A. and Bjorkman, P.J. (2000) Crystal structure of the hereditary hemochromatosis protein HFE complexed with transferrin receptor. *Nature* **403**:46-53.

Chapman, T.L., Heikema, A.P., West, A.P. and Bjorkman, P.J. (2000) Crystal structure and ligand binding properties of the D1D2 region of the inhibitory receptor LIR-1 (ILT2). *Immunity* **13**:727-736.

Herr, A.B., White, C.L., Milburn, C., Wu, C. and Bjorkman, P.J. (2002) Bivalent binding of IgA1 to FcaRI suggests a mechanism for cytokine activation of IgA phagocytosis. Manuscript submitted.

Kennedy, M.W., Heikema, A.P., Cooper, A., Bjorkman, P.J. and Sanchez, L.M. (2001) Hydrophobic ligand binding by ZAG, a soluble fat-depleting factor related to class I MHC proteins. *J. Biol. Chem.* **276**:35008-35013.

Liu, Y., Chirino, A.J., Leteux, C., Feizi, T., Misulovin, Z., Nussenzweig, M.C. and Bjorkman, P.J (2000) Crystal structure of the cysteine-rich domain of mannose receptor complexed with a sulfated carbohydrate ligand. *J. Exp. Med.* **191**:1105-1116.

Liu, Y., Misulovin, Z. and Bjorkman, P.J. (2001) Molecular mechanism of sulfated carbohydrate recognition by the cysteine-rich domain of mannose receptor. *J. Mol. Biol*. **305**:481-489.

Martin, W.L., West, A.P., Gan, L. and Bjorkman, P.J. (2001) Crystal structure at 2.8 Å of an FcRn/heterodimeric Fc complex: Mechanism of pH-dependent binding. *Mol. Cell* **7**:867-877.

O'Callaghan, C.A., Cerwenka, A., Willcox, B.E., Lanier, L.L. and Bjorkman, P.J. (2001) Molecular competition for NKG2D: H60 and RAE1 compete unequally for NKG2D with dominance of H60. *Immunity* **15**:201-211.

Ramalingam, T.S., West, A.P., Lebrón, J.A., Nangiana, J.S., Hogan, T.H., Enns, C.A. and Bjorkman, P.J. (2000) Binding to the transferrin receptor is required for endocytosis of HFE and regulation of iron homeostasis. *Nature Cell Biol.* **2**:953-957.

Vogt, T.M., Blackwell, A.D., Giannetti, A.M., West, A.P., Bjorkman, P.J. and Enns, C.A. (2002) Heterotypic interactions between TfR2 and TfR. Manuscript submitted.

West, A.P. and Bjorkman, P.J. (2000) Crystal structure and immunoglobulin G binding properties of the human MHC-related Fc receptor. *Biochemistry* **39**:9698-9708.

West, A.P., Bennett, M.J., Sellers, V.M., Andrews, N.C., Enns, C.A. and Bjorkman P.J. (2000) Comparisons of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE. *J. Biol. Chem*. **275**:28135-28138.

West, A.P. Jr., Llamas, L.L., Snow, P.M., Benzer, S. and Bjorkman, P.J. (2001) Crystal structure of the ectodomain of *methuselah*: A *Drosophila* GPCR associated with extended life span. *Proc. Natl. Acad. Sci. USA* **98**:3744-3749.

West, A.P. Jr., Giannetti, A.M, Herr, A.B., Bennett, M.J., Nangiana, J.S., Pierce, J.R., Snow, P.M. and Bjorkman, P.J. (2001) Mutational analysis of the transferrin receptor reveals overlapping HFE and transferrin binding sites. *J. Mol. Biol*. **313**:385-397.

Professor of Chemistry and Biology: Judith L. Campbell
Associate: Elizabeth Bertani
Member of the Professional Staff: Martin Budd, Piotr Polaczek
Research Fellows: Susanna Boronat, Shaune Edwards, Osamu Imamura, Caroline Li, Tao Weitao
Graduate Students: Wonchae Choe, Isabelle Lesur
Research and Laboratory Staff: Jules Chen

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Summary: A hallmark of cancer cells, in addition to uncontrolled proliferation, is genomic instability, which appears in the form of chromosome loss or gain, gross chromosomal rearrangements, deletions, or amplifications. The mechanisms that suppress such instability are of the utmost interest in understanding the pathogenesis of cancer. Our lab studies the components of the DNA replication apparatus that promote genomic stability, primarily using yeast genetics and biochemistry.

Several years ago, Rajiv Dua in the laboratory discovered that DNA polymerase ε, one of four essential DNA polymerases in yeast, had not one, but two essential functions. Deletion of the polymerase domain left the cells viable because another polymerase activity could substitute. Conversely, deletion of the remaining, non-catalytic half of the protein was lethal. Shaune Edwards in the laboratory carried out a two-hybrid screen for proteins that interact with the enigmatic C-terminal region of pol ε in order to discover its function. She found that pol ε interacts with Trf4, a protein involved in establishing cohesion of sister chromatids during passage of the replication fork. She has gone on to develop evidence that the essential function of the C terminus of pol ε is to aid in establishing efficient sister chromatid cohesion during S phase. Another postdoctoral fellow in the laboratory, Caroline Li, has characterized the Trf4 protein. She has shown that it encodes a previously unknown DNA polymerase and that it stimulates the activity of pol ε dramatically. Interestingly, the protein also contains polyA polymerase activity. Future studies are aimed at defining the mechanism by which these two proteins regulate interaction of the replisome with the cohesin complex, the glue that holds the chromosomes together, and how failure of cohesion leads to genomic instability.

At least seven human diseases characterized by cancer predisposition and/or premature aging are correlated with defects in genes encoding DNA helicases. The yeast genome contains 134 open reading frames with helicase motifs, only eight of which have been characterized. Martin Budd in our laboratory identified the first eukaryotic helicase essential for DNA replication, Dna2. He showed by interaction studies that it was a component of the machine that is required for accurate processing of Okazaki fragments during lagging-strand DNA replication. Enzymatic studies to elucidate the sequential action of the DNA polymerase, helicase, and nuclease required for this processing is an ongoing mechanistic biochemistry project in the laboratory.

Stimulated by various reports in the literature implicating Dna2 in telomere biogenesis and structure, Wonchae Choe made the interesting observation that the bulk of Dna2 is localized to telomeres and that this localization is dynamic. During G1 and G2 phases of the cell cycle, Dna2 is at telomeres. During S phase Dna2 is present on the replicating chromatin. Current studies are aimed at defining the genes that regulate the localization. In addition to defects in replication, *dna2* mutants are also very sensitive to agents that induce double strand breaks (DSBs). Osamu Imamura has shown that Dna2 is mobilized from telomeres in response to the induction of double strand breaks. He is carrying out experiments to test the model that Dna2 delocalization from telomeres is part of the signaling system that induces the DNA damage and S-phase checkpoints, as has also been suggested for yKU, a protein involved in non-homologous end joining and in stabilizing telomeres.

One model of cellular aging suggests that accumulation of DNA damage leads to replicative senescence. Most endogenous damage occurs during S phase and leads to DSBs due to collapsed replication forks. At least three human diseases of premature aging or cancer predisposition - Werner, Bloom, and Rothmund-Thomson - are caused by defects in helicases similar to Dna2. Martin Budd and Laura Hoopes found that *dna2* mutants have a significantly reduced life span. Microarray analysis by Isabelle Lesur shows that the *dna2* mutants age by the same pathway as wild-type cells; they just age faster. Interestingly, the human Bloom gene (though not human Werner or Rothmund-Thomson) complements the replication defect of *dna2* mutants. Future work will take advantage of the yeast system to further delineate the role of BLM protein in mammalian cells. The work of Tao Wei in the lab suggests that instability of repetitive DNA, such as the ribosomal locus and telomeric DNA, is a major source of genomic instability in the aging *dna2* mutants.

228. Non-magnetic variants of magnetotactic bacteria

Elizabeth Bertani, Albert Nguyen[1], Cody Nash[2]

Magnetotactic bacteria produce magnetite-containing structures called magnetosomes - single-domain crystals of magnetite enclosed in protein-lipid membranes. One possibility is that magnetite forms when iron oxides are transported into preformed magnetosomal membranes. In an effort to understand this process better, we are attempting to prepare intact magnetosomal membranes that contain little or no magnetite and to set up an *in vitro* system for the formation of magnetite within the membranes. In preliminary attempts to produce such membranes, we have found that one magnetotactic strain, AMB-1, stops making magnetite when grown in the presence of excess air. Such non-magnetic strains grow faster and to higher density than the magnetite-containing parental strain. In addition, they are less sensitive to UV light. So far we have been unable to demonstrate a return to magnetite formation by culturing the strains in the

absence of air, suggesting the permanent loss of some critical factor. We are investigating if the critical factor might be carried by one of the bacteriophage-like or transposon-like structures that total genome analysis has revealed to be present in these magnetotactic bacteria. The phage and transposon sequences also represent possibilities for the lateral transfer of genes involved in magnetite formation to other strains.

[1]*Undergraduate, California Institute of Technology*
[2]*Division of Geology and Planetary Science*

229. Role of the replication protein Cdc6 in the progression of the cell cycle

Susanna Boronat, Judith L. Campbell

The replication protein Cdc6p is synthesized at the end of mitosis, binds to the chromosomally-bound ORC, and in turn enables the subsequent binding of Mcm proteins at the replication origins during G1 to form pre-replicative complexes (preRCs). During late G1-phase, preRCs are activated by Clb5,6/Cdc28 and Dbf4p/Cdc7p protein kinases leading to the onset of S phase. At this stage, the preRCs are lost from origins and cannot reassemble until passage through the next mitosis, since the Clb/Cdc28 kinases block preRC formation. The levels of Cdc6p fluctuate during cell cycle: Cdc6p is synthesized at the end of mitosis, and is subject to degradation at the onset of S phase.

Cdc6p contains six potential target sites for the Cdc28 protein kinase, three at the N-terminus and three at the C terminus. The mutation of all these sites, a subset of them, or just one site at the C terminus (site 6), into non-phosphorylatable sites results in the stabilization of Cdc6p.

We and others have reported previously that Cdc6p ectopic expression and specially the expression of phosphorylation site mutants, produces a delay in mitosis, specifically at the metaphase to anaphase transition. We had proposed that this delay could be explained by two different kinds of explanations, although not mutually exclusive. One possible explanation is that Cdc6p ectopic expression triggers or mimicks a checkpoint that arrests cells at the metaphase to anaphase transition. We have shown that no one of the checkpoint mutants that we have tested can suppress the mitotic delay, assayed by monitoring the levels of the Pds1 protein. This result strongly suggested that Cdc6p ectopic expression is not triggering the DNA replication or the spindle checkpoint.

The second kind of explanation relies on the interaction of Cdc6p with Cdc28p. The activation of APC both at the metaphase to anaphase transition and at the exit of mitosis requires active Cdc28 kinase. It has been shown in our laboratory and by others that Cdc6p may inhibit Cdc28 kinase activity. Therefore, it is possible that Cdc6p ectopic expression inhibits Cdc28p and this results in decreased APC activity. The results that we had previously obtained did not rule out the possibility that the G2/M delay was due to an inhibition of Cdc28 kinase activity that we could not detect with our experimental approach (using H1 as a substrate in *in vitro* phosphorylation assays). But, at the same time, these results strongly suggested that there had to be another mechanism other than the direct inhibition of Cdc28p by binding to the Cdc6p, because some of our phosphorylation site mutants did not bind to Cdc28 and were still capable of producing a mitotic delay.

To better understand the role of the interaction between Cdc28p and Cdc6p in the mitotic delay (APC inhibition, firm the existence of an additional mechanism for this inhibition.

230. Role of Cdc55p in the inhibition of APC by Cdc6p ectopic expression

Susanna Boronat, Judith L. Campbell

One of the regulatory B subunits of the PP2A phosphatase, Cdc55p, is involved in the spindle checkpoint pathway and it interacts genetically with *MIH*1 and *SWE*1, both regulators of the Cdc28p kinase activity. Interestingly, human Cdc6 protein has been shown to interact with PR48, a novel regulatory subunit of human PP2A. This regulatory subunit has been shown to have a role in DNA replication.

To determine if Cdc55p has a role in the inhibition of APC in *S. cerevisiae*, as has been described for the E4orf4 protein of human adenovirus, we have disrupted the *CDC55* gene in yeast strains expressing wild-type and phosphorylation-site mutants of Cdc6 and containing HA-tagged Pds1p. We have performed α-factor arrest and release experiments and monitored the levels of Pds1p during one cell cycle after the release. As opposed to wild-type strains expressing stable Cdc6p mutants, *cdc55*Δ strains show a normal kinetics of Pds1p appearance and degradation, indicating that *cdc55*Δ can suppress the inhibition of Pds1 degradation. It has been shown that *cdc55*Δ strains have increased levels of Swe1p, a Cdc28p inhibitory kinase. If even under these circumstances (more Cdc28p inhibition) APC inhibition is bypassed, it strongly suggests that Cdc28p kinase inhibition is not the only mechanism for the mitotic delay.

Since it has been shown that a human PP2A regulatory subunit interacts with human Cdc6, we have tested if Cdc55p interacts with yeast Cdc6p. We have constructed yeast strains that express HA-tagged Cdc55p and myc-tagged wild-type and mutant Cdc6p. We have performed co-immunoprecipitation assays that clearly show a specific interaction between Cdc55p and Cdc6p. Not all the Cdc6p mutants interact with Cdc55p with the same affinity. Interestingly, a Cdc6p mutant with a truncated N terminus that does not show any cell cycle defect exhibits a very strong interaction with Cdc55p.

231. Role of Cdc28 in Cdc6-inhibition of the cell cycle

Susanna Boronat, Judith L. Campbell

Our H1 phosphorylation assays did not show any decreased Cdc28 protein kinase activity in strains expressing stable mutants of Cdc6p that did produce a mitotic delay. However, it has recently been reported that ectopic expression of a stable phosphorylation site mutant of Cdc6p does result in a strong inhibition of Cdc28p. The authors used a very appropriate assay for the Cdc28p kinase activity because they used a physiological substrate that is phosphorylated in mitosis, the B subunit of the polymerase α. The authors determined the phosphorylation state of this protein by looking at the electrophoretic

mobility shift produced by changes in phosphorylation. We have used the same approach for our own mutants. To do that, we HA-tagged the *POL12* gene coding for the B subunit of polymerase α in strains expressing wild-type and mutant Cdc6p. We have only detected a very slight inhibition of Cdc28p kinase in strains expressing one particular phosphorylation site mutant, at site 6. However, no one of the other mutants that produce a mitotic delay resulted in Cdc28p kinase inhibition. Interestingly, those Cdc6p mutants not inhibiting Cdc28p but producing mitotic delay are the same that do not interact with Cdc28p. These results further indicate that APC inhibition is not exclusively due to Cdc28p kinase inhibition.

232. Interaction between Cdc6p and Cdc16p

Susanna Boronat, Judith L. Campbell

In the particular case of the E4orf4 of human adenovirus it has been shown that this protein inhibits the APC and it can interact with one of its subunits, Cdc16p. We have tested if that was also true for yeast Cdc6p, by tagging Cdc16p with the HA epitope and expressing it in our strains with wild-type and mutant Cdc6p. We have performed co-immunoprecipitation assays and the preliminary results indicate that Cdc6p interacts with Cdc16p. We are currently analyzing the possibility that the interaction between Cdc6p and Cdc16p is Cdc55p dependent.

These results suggest a direct role of Cdc6p in APC inhibition. We are interested in understanding the mechanism of this inhibition, but most importantly the biological significance of these interactions.

233. Interaction of Dna2 protein with Ddc2 and YHR122w proteins

Martin Budd, Osumu Imamura, Judith Campbell

Using tandem-affinity purification and mass spectrometry, Cellzone has identified protein-protein interactions for 1,739 *Saccharomyces cerevisiae* genes (1). Two genes were found to interact with Dna2 protein, YHR122w and Ddc2. YHR122w is an essential open reading frame coding for a 26 kd protein with unknown function. The deletion mutant arrests at multiple stages of the cell cycle, suggesting it is not a classical cdc mutant. YHR122w has been tagged with the HA epitope and is being retested for an interaction with Dna2 protein. The genetic interaction with Dna2 can be expressed by identifying conditional mutants of YHR122w and testing for genetic interactions with *dna2* mutants. Ddc2 protein is required for the S-phase and G2/M-phase checkpoint. Ddc2 protein interacts with the Mec1 protein kinase and is required for Mec1 checkpoint function. We have tagged Ddc2 with the HA and MYC epitopes and we are examining a physical interaction with Ddc2 and Dna2. Since Ddc2 and Mec1 proteins interact, we are testing whether Mec1 protein phosphorylates Dna2 protein. Dna2 mutants can activate the checkpoint without creating DNA damage that causes lethality. The viability of dna2 mutants is enhanced in *rad9*-and *mec1*-deficient strains. Dna2 protein is localized at the telomeres during G1 and G2 phases. After DNA damage dna2 relocates from the telomeres into diffuse sites in the nucleus. Dna2 with ddc2 may perform a function in checkpoint control by binding to the telomere when the checkpoint is off and leaving the telomere when the checkpoint is on.

Reference

(1) Gavin, A. *et al.* (2002) *Nature* **415**:141-147.

234. The role of DNA2 in *Xenopus* DNA replication

Jules Chen, Peter Snow

Dna2 is a DNA helicase and nuclease protein involved in DNA replication. Dna2 contains a N-terminal nuclease motif and a C-terminal helicase motif. The helicase motif includes the Walker A and Walker B box and a DNA-dependent ATPase. Previously in the lab, we cloned and sequenced the DNA2 gene from *Saccharomyces cerevisiae* and *Xenopus*. *Xenopus* Dna2 protein was found to be 32% identical to yeast Dna2 protein. Experimental data supports that the Dna2 gene of *Xenopus* is a functional as well as a structural homolog of the yeast protein.

Replication protein A (RPA) is a single-stranded DNA-binding protein that is essential for DNA replication. RPA is a heterotrimeric single-stranded DNA binding protein that is conserved among all eukaryotes. The largest subunit, RPA70, binds to DNA during DNA replication to allow binding of DNA polymerase and replication of the single-stranded DNA.

Immunofluorescent microscopy experiments with *Xenopus* egg extract demonstrated the appearance of foci containing both RPA70 and DNA2. RPA70 foci appear 30 minutes after the incubation of sperm DNA in *Xenopus* egg extract. DNA2 foci punctually appear following the development of RPA70 foci. Furthermore, immuno-depletion of DNA2 from *Xenopus* egg extract abolishes the appearance of RPA70 foci. These results correlate the function between DNA2 and RPA70.

Xenopus Dna2 ATPase and nuclease mutants are being expressed and purified from insect Sf9 cells. These mutations are expected to be dominant negative. Addition of these mutant proteins to *Xenopus* replication egg extracts should inhibit DNA replication and/or the formation of Dna2/RPA70 foci.

235. The human Bloom syndrome gene *BLM* suppresses the DNA replication defect of yeast *dna2* mutants

Osamu Imamura, Judith L. Campbell

Deficiency in a helicase of the RecQ family is found in at least three human genomic instability disorders associated with cancer predisposition and/or premature ageing. The RecQ helicases encoded by the *BLM*, *WRN* and *RTS* genes are defective in Bloom, Werner and Rothmund-Thomson syndromes, respectively. *Saccharomyces cerevisiae* has a single RecQ family member, *SGS1*. Deletion of *SGS1* causes hyper-recombination, premature aging, and a defect in the intra-S-phase DNA damage checkpoint. *SGS1* interacts genetically with several yeast helicase-encoding genes, including one that we have been studying recently, *DNA2*. DNA2p is thought to be involved in maturation of Okazaki fragments. Mutation of *DNA2* causes premature aging and increased homologous recombination at the

rDNA loci. In addition, *sgs1dna2* double mutants grow very slowly. Thus, it has been speculated that the RecQ helicases and *DNA2* may encode redundant functions. We were, therefore, interested whether the human RecQ helicase genes could suppress any of the phenotypes of *dna2* mutants. The *hBLM*, *mWRN* and *hRTS* were each cloned into the yeast centromere vector pRS316 under control the *Gal* promoter. *hBLM,* but not *mWRN* or *hRTS,* suppressed the temperature-sensitive growth defect of a *dna2-1*. The *hDNA2* also complemented the temperature-sensitive growth defect of a *dna2-1*. To further test for specificity, a mutation that has been shown to inactivate the hBLM helicase domain, K695T, was expressed in the same way in the *dna2-1* strain. There was no complementation at 37°C. Thus, the helicase activity of hBLM is essential to suppress the *dna2-1* defect. Complementation of a DNA replication mutant provides a novel link between DNA replication defect and Bloom syndrome. Conversely, the ability of hBLM to rescue the *dna2* mutant sheds further light on the role of helicase activity of DNA2 and/or the type of damage encountered in its absence in yeast.

236. Use of gene expression profiling to identify pathways involved in the premature aging of *dna2-1*, a *Saccharomyces cerevisiae* DNA replication mutant

Isabelle Lesur, Judith L. Campbell

Aging is a process in which individuals undergo a progressive decline in vitality, leading to death. The mechanisms involved in aging are complex and, for the most part, unknown. Recently, there has been interest in using yeast as a model system for discovering pathways that affect aging. We recently demonstrated that *DNA2*, which encodes a yeast helicase/nuclease involved in DNA replication and repair, is required for normal life span. To further establish that the life span of *dna2* mutants is shortened due to acceleration of normal aging and to try to identify the processes leading to early death, we compared the gene expression profiles of wild type and dna2-1 in young and older cells isolated by elutriation. We found that 761 genes showed similar aging-related elevation and 201 genes similar aging-related decrease in expression in the two strains. As recently documented by others, there is a shift away from glycolysis toward gluconeogenesis and energy storage during aging, and we find that this is also true of the mutant. This adds to previous evidence suggesting that normal aging is leading to cell division arrest in the mutants. Also, we observe induction of several DNA repair pathways, suggesting that aging is accompanied by increased DNA damage in both wild type and mutant. Notably, however, we find that a number of genes involved in repair of double-strand breaks (*RAD51, 54, 55, 59, RHC18, MUS81*), which are induced in wild type, are either not induced or only slightly elevated in the *dna2* mutant. Thus, *DNA2* may be required for damage-induced transcription of these genes.

237. Physical and biochemical interactions of two essential *Saccharomyces cerevisiae* DNA polymerases

Caroline Li, Peter Snow, Judith Campbell

Four essential nuclear DNA polymerases in *Saccharomyces cerevisiae* include Pol α, β, ε (Pol2p, Dpb2p, Dpb3p,and Dpb4p) and σ (Trf4p and Trf5p). Trf4 and Pol2 have DNA polymerase activity, are involved in DNA damage repair, and play a role in sister chromatid cohesion. During the course of the last year, our group has made major advances in linking pol ε to sister chromatid cohesion.

Previous two-hybrid experiments in our lab showed that the C terminus of Pol2p (amino acids 1265-2222) interacts with Pol σ. To map the Trf5p interaction region with Pol2p more closely, a two-hybrid assay was conducted using a set of *pol2* mutants carrying ten sequential amino acid deletions spanning the C-terminal zinc-finger region (amino acids 2103-2222). Some mutants of Pol2p abolished interaction with Trf5, whereas others reduced the interaction. Since the mutations also can affect stability of the holoenzyme, we cannot be sure that this region represents an interface between Trf5p and Pol2p. Coimmunoprecipitation studies showed that Pol2p physically interacts with Trf4p and Trf5p.

Saccharomyces cerevisiae Trf4p and Trf5p were expressed and purified from insect cells. Biochemical assays revealed that Trf4p and Trf5p have limited DNA polymerase activity. However, the DNA polymerase activity of Pol ε was dramatically stimulated in the presence of Trf4p or Trf5p. Surprisingly, equal stimulation was seen in a Trf4p mutant with a mutation in the polymerase active site. No DNA polymerase activity was observed in the presence Pol ε, Trf4, and *N*-ethylmaleimide (an inhibitor of Pol ε but not Trf4). These observations suggest a structural role of Trf4p rather than catalytic. We are investigating the model that Trf4/5 allow Pol ε to replicate past blocks in the template, such as those due to base damage or bound proteins. Fission yeast Cid13 was recently found to have poly(A) polymerase activity. Since Cid13 and Trf4/5 are members of the same nucleotidyltransferase family, we are currently investigating whether Trf4/5 have poly(A) polymerase activity.

238. DNA double-strand breaks caused by replication arrest at the replication fork barrier in eukaryotic cells

Tao Weitao, Martin Budd, Judith L. Campbell

Studies on a prokaryotic model *Escherichia coli* show that replication arrest induces formation of DNA double-strand breaks (DSB). However, the direct evidence in eukaryotic systems is lacking. Here we used yeast rDNA model to provide direct evidence for this hypothesis in eukaryotic cells. Using two-dimensional (2D) gel and DSB analysis, we found that a naturally occuring replication pause of *Saccharomyces cerevisiae* chromosome at a replication fork barrier (RFB) in the rDNA region accompanies with occurrence of DBSs. The replication fork stalling and DSB formation are dependent on FOB1 and DNA replication. We therefore propose that replication fork pausing at the RFB causes DSBs in yeast

cells. To support this, we found that the fork stalling increased in the dna2-2 mutant with a mutation in the replicative DNA helicase domain. DBSs were also generated at a elevated level during S phase. A mutation disrupting FOB1 that alleviates the fork stalling at the RFB and DBS formation suppresses the phenotypes of the elevated replication pausing and DBS formation of the dna2-2 mutant. The *fob1* mutation also suppresses sensitivity of the *dna2-2* mutant to bleomycin, a DSB-inducing agent and affects telomere lengthening of the dna2-2 mutant. These imply general effects of *FOB1* and *DNA2* not only on replication progression and DSB formation but also global chromosome stability.

239. Concordant action of Sgs1 and Dna2 at the Fob1-dependent replication fork barrier in yeast

Tao Weitao, Martin Budd, Judith L. Campbell

We used the yeast rDNA model to study mechanisms of combined action of DNA replication and recombination helicases on Fob1-dependent replication fork barrier (RFB) using two-dimensional (2D) gel analysis. We studied two yeast DNA helicases of potential importance to human BLM, which is involved in resolving abnormal DNA structure. Dna2, encoded by an essential gene, is a replication helicase/nuclease, which is required for DNA lagging-strand replication and post-replication repair. Our work has led to several important discoveries.

(I) In the *dna2* mutant, lesions including nicks and single-stranded gaps were detected near the RFB.

(II) In the *sgs1* mutant, fork stalling, converging and formation of Holliday junction were exacerbated at the RFB.

(III) Mutations inactivating both *DNA2* and *SGS1* cause even severe replication pausing and growth defect. The *dna2* mutant was also synthetically lethal with a mutation in *RRM3*, encoding a DNA helicase.

(IV) The *fob1* mutation suppresses the fork stalling and hyper-recombination of the single *dna2-2* or *sgs1* and *dna2-2 sgs1* double mutants.

(V) Initiation frequency from the proximal rDNA ARS is not affected in the *dna2-2* mutant but is reduced or in the *fob1* mutant, suggesting that Fob1 is a positive or stabilizing factor for initiation from rDNA ARS. It is plausible that the deteriorating initiation contributes to the suppression of hyper-replication pausing by Fob1.

(VI) The *fob1* mutation suppresses the growth defect of the *dna2 sgs1* double mutant. Taken together, *Sgs1* and *Dna2* have a concordant action on the RFB, but presumably via different mechanisms.

Publications

Choe, W., Budd, M., Imamura, O., Hoopes, L.M. and Campbell, J.L. (2002) Dynamic localization of an Okazaki fragment processing protein suggests a novel role in telomere replication. *Mol. Cell. Biol.* **22**:2002-2017.

Dua, R., Levy, D.L., Li, C. and Campbell, J.L. (2002) *In vivo* reconstitution of *Saccharomyces cerevisiae* DNA polymerase ε in insect cells: Purification and characterization. *J. Biol. Chem.* **277**:7889-7896.

Hoopes, L.M., Budd, M., Choe, W., Weitao, T. and Campbell, J.L. (2002) Effects of mutations in DNA replication genes on yeast lifespan. *Mol. Cell. Biol.* **22**:4136-4146.

Jang, S.W., Elsasser, S., Campbell, J.L. and Kim, J. (2001) Identification of Cdc6 protein domains involved in interaction with Mcm2 protein in budding yeast cells. *Biochem. J.* **354**:655-661.

Assistant Professor: David C. Chan
Postdoctoral Scholars: Hsiuchen Chen, Takumi Koshiba
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Summary: Diverse biological systems, including viruses, cells, and organelles, are enclosed within lipid membranes that normally serve to compartmentalize the systems and distinguish their contents from the environment. Under certain circumstances, however, these membrane-bound systems undergo membrane fusion, in which the lipids of two compartments fuse and the internal contents ultimately mix. In general, these fusion events are multi-step processes, involving recognition of two membrane surfaces, membrane apposition, and finally, lipid and content mixing. Such membrane fusion events are central to many fundamental cellular processes, including entry of enveloped viruses into cells during infection, entry of sperm into an egg during fertilization, and fusion of organelles during protein trafficking and organelle biogenesis. Using cell biological, biophysical, and genetic approaches, our lab is studying the mechanisms through which these membrane fusion events occur.

Regulation of mitochondrial dynamics

Our major research focus is on the regulation of mitochondrial dynamics. Mitochondria are dynamic organelles that undergo cycles of homotypic fusion and fission. Such membrane fusion and fission events play important roles in controlling organelle number, subcellular distribution, morphology, and ATP production. In some cells, fusion of numerous mitochondria into a well-organized reticulum is thought to enable transmission of mitochondrial membrane potential, thereby facilitating ATP generation to active regions of the cell. In some cases, mitochondrial fusion is developmentally regulated; for example, during insect spermatogenesis, the mitochondria of immature spermatids aggregate and fuse into giant mitochondria. Such regulated mitochondrial fusion is likely required in order to accommodate the changing metabolic state of the cell. Recent experiments in flies and budding yeast have demonstrated that developmentally regulated fusion of mitochondria is necessary for respiratory function and have identified the mitochondrial GTPase, Fzo, as an essential player. In addition, genetic studies in yeast and *C. elegans* have identified a second GTPase, Dnm1, that plays a central role in fission of mitochondria.

Although the importance of mitochondrial fusion in lower eukaryotes is well documented, its role during mammalian development remains unclear. Accordingly, we have placed a major emphasis on addressing this issue through the analysis of mice deficient in homologs of Fzo (see abstract by Hsiuchen Chen). This work should reveal the role of mitochondrial fusion in tissue development and bioenergetics. Furthermore, these studies may also lead to mouse models of human mitochondrial diseases, a diverse group of diseases characterized by defective mitochondrial function.

Genetic studies have uncovered Fzo as a central molecule in the fusion of mitochondria, but its mechanism of action is unknown. It may act as a truly fusogenic molecule, analogous to viral envelope proteins that directly mediate membrane fusion. Alternatively, it may act as a regulatory protein, directing assembly of a fusion machinery in a GTP-dependent manner. We are currently performing extensive structure-function analyses of Fzo to address these issues (see abstracts by Scott Detmer and Erik Griffin). We have also begun attempts to genetically and biochemically characterize proteins that interact with Fzo in order to identify other proteins involved in mitochondrial fusion (see abstract by Scott Detmer).

Yeast Dnm1p and its mammalian homolog, Drp1 (dynamin-related protein), are essential in mediating mitochondrial membrane fission. These GTPases are structurally related to dynamin, a well-studied protein involved in fission of the plasma membrane during endocytosis. We have begun biochemical studies on Dnm1p and Drp1 with the goal of understanding its mechanism of action (see abstract by Tobias Rosen).

HIV entry

In a second line of research, we are continuing our investigations of membrane fusion by viral envelope proteins. A key step in the life cycle of enveloped viruses is fusion of viral and host cell membranes. A virally encoded glycoprotein, gp160, is responsible for mediating the entry process of HIV-1, the etiological agent of Acquired Immunodeficiency Syndrome (AIDS). The envelope precursor, gp160, is cleaved to form the subunits gp120 and gp41. gp120 directs target cell recognition, while gp41 mediates the merging of viral and cellular membranes. gp41 is composed of several distinct domains, including a hydrophobic fusion peptide, two coiled-coil domains (termed N- and C-terminal helices), a membrane-spanning region, and a cytoplasmic tail.

The current model for HIV membrane fusion invokes a series of conformational changes in the gp120/gp41 complex. Interaction between gp120 and cellular receptors liberates the gp41 fusion peptide from its native conformation and allows its insertion into the host cell membrane. A transient species termed the prehairpin intermediate is created, in which the N-terminal helices form a trimeric coiled-coil but do not interact with the C-terminal region. Subsequently, a hairpin structure is generated, in which the C-terminal helices pack in an anti-parallel manner around the trimeric N core forming a fusion-active 6-helix bundle. This N-C interaction brings the viral fusion peptide, anchored to the host-cell

membrane, and the transmembrane domain, associated with the virion, into close proximity. In a process that remains poorly defined, fusion of viral and cellular membranes follows. A mechanistic understanding of HIV membrane fusion may lead to new strategies to inhibit HIV entry into human cells.

To test this model of HIV entry, we are determining the mechanism through which particular peptide inhibitors work to prevent gp41-mediated membrane fusion. Some of these peptide inhibitors bind to fusion intermediates of gp41, and an understanding of their mode of action can reveal sequential steps in the fusion pathway (T. Koshiba). In addition, we are characterizing a series of gp41 mutants that fail to fuse in order to better understand how formation of the 6-helix structure leads to membrane fusion (T. Suntoke).

240. Analysis of mitochondrial fusion in mouse development and adult physiology

Hsiuchen Chen

Mice contain two Fzo homologs, termed Mfn1 and Mfn2, that are highly similar to yeast and *Drosophila* Fzo. Both mitofusins are broadly expressed throughout development and adulthood. As expected for proteins involved in mitochondrial fusion, both Mfn1 and Mfn2 are localized to mitochondria. To determine the role of mitochondrial fusion in mammalian development, we have generated mice deficient in each of the two Fzo homologs.

Phenotypic analysis of the Mfn knockouts has determined that both mitofusins are required for normal mouse development, because homozygous mutant mice die in mid-gestation between embryonic days 10.5-11.5. The cause of lethality in Mfn2-deficient mice has been identified as a placental defect. Specifically, the trophoblast giant cell layer is markedly reduced in homozygous mutants as compared to those of wild-type and heterozygous littermates. Trophoblast stem cells and differentiated giant cells both display fragmented mitochondria, a morphology consistent with loss of mitochondrial fusion. In addition, mutant cells display a loss of membrane potential in some mitochondria. It is likely, therefore, that Mfn2-deficiency does indeed disrupt mitochondrial fusion and subsequently, mitochondrial function.

Since the embryonic lethality associated with Mfn-deficiency precludes analysis of mitochondrial fusion in adult tissues, we are generating conditional knockout mice for both mitofusins. Tissue-specific knockouts will then be used to analyze the role of mitochondrial fusion in skeletal muscle, cardiac muscle, the central nervous system, and endocrine organs - systems which most often manifest clinical abnormalities in human mitochondrial diseases.

241. Structure-function analysis of murine Mfn1 and Mfn2

Scott Detmer

At present, we have almost no understanding of how Fzo proteins function from a mechanistic perspective. It may act as a directly fusogenic molecule, using energy from GTP hydrolysis or coiled-coil formation to appose membranes and mediate fusion. In such a model, it would play a role analogous to viral fusion proteins, which contain helical hairpins that are thought to appose lipid membranes during viral entry. Alternatively, Fzo may act as a regulatory protein that assembles a separate fusion machinery in a GTP-dependent manner. In either case, it is likely that the study of Fzo will yield novel insights into membrane fusion, since mitochondrial fusion is a unique process that fuses two sets of bilayer membranes.

To better understand the role of Mfn1 and Mfn2 in mitochondrial fusion, we are performing a systemic structure-function analysis. Cells derived from Mfn1-/- and Mfn2-/- mice provide an ideal experimental system for such analysis. Whereas wild-type cells have reticulated and tubular mitochondria, the mitochondria of cells lacking Mfn1 are small, round and individual. A similar mitochondrial morphology is observed in cells lacking Mfn2. Expression of epitope-tagged Mfn1 and Mfn2 in the respective mutant cell lines completely restores wild-type mitochondrial morphology. In addition, Mfn1 can partially rescue the mitochondrial morphology defect in Mfn2-deficient cells, as can Mfn2 in Mfn1-deficient cells. We are using this system to test Mfn1 and Mfn2 mutants for their ability to localize to the mitochondria and to restore wild-type mitochondrial morphology.

Both Mfn1 and Mfn2 contain an N-terminal GTPase domain and two predicted coiled-coil domains separated by two transmembrane domains. Point mutations in the GTPase domain predicted to prevent GTP hydrolysis are severe loss-of-function alleles in both Mfn1 and Mfn2, suggesting that GTPase activity is required for Mfn function. Proline substitutions and alanine substitutions in the "a" and "d" positions in the predicted coiled-coil regions are expected to disrupt oligomerization by disrupting the helical nature of the domain and the hydrophobic interface, respectively. We are testing both such mutations in Mfn1 and Mfn2, as well as various deletion mutants.

242. Biochemical characterizations of murine Mfn1 and Mfn2

Scott Detmer

To understand the interactions of Mfn1 and Mfn2, we are examining these proteins biochemically. A first step is to address whether Mfn1 and Mfn2 interact homo- and heterotypically *in vivo*. We are using a differential tagging method in which Mfn constructs are tagged with either Myc or HA tags and examined for intermolecular interactions by co-immuniprecipitation. We have found that Mfn1 and Mfn2 interact both homo- and heterotypically. We are beginning to examine whether loss-of-function and dominant mutations in Mfn1 and

Mfn2 identified in Mfn structure-function analysis maintain these interactions both with themselves and with wild-type Mfn1 and Mfn2. We will use this system to identify domains of Mfn1 and Mfn2 required for intermolecular interactions.

To identify unknown interacting partners of Mfn1 and Mfn2 we are using a similar immunoprecipitation approach. We have generated cell lines in which endogenous Mfn1 and Mfn2 have been replaced with Mfn1 and Mfn2 containing a C-terminal Myc epitope. We will immunoprecipitate Mfn using an anti-Myc antibody. These samples will be analyzed by SDS-PAGE and autoradiography and promising interacting bands will be identified using N-terminal sequencing and mass spectrometry based peptide mapping.

243. Structure-function analysis of yeast Fzo1p

Erik Griffin

To understand the mechanism of Fzo1p action, it is necessary to identify its critical domains. Our structure/function analysis has defined several critical regions in Fzo1p required for mitochondrial fusion. The loss-of-function point mutants resulting from this analysis are currently being characterized in more detail using both *in vitro* and cell-based assays for GTPase activity, oligomerization, and mitochondrial fusion as well as genetic assays for allelic complementation. Purified recombinant fragments of Fzo1p are also being characterized biochemically to verify our *in vivo* data. This approach should allow us to define the role each region of Fzo1p plays in mitochondrial fusion and to generate a model for its role in mitochondrial fusion.

Mitochondrial fusion is likely to be a multi-step process in which mitochondria first associate through specific protein interactions and later proceed to membrane fusion. Constructs of Fzo1p lacking the GTPase domain cause severe clumping of individual mitochondria. This phenotype may represent mitochondria which recognize and associate with each other but are unable to complete the fusion reaction. Mutants which do not cause clumping have been identified and implicate two protein interaction domains in this process. Immunoprecipitations and screens for genetic suppressors of this mutant are also being performed to identify interacting proteins.

244. A biochemical and structural analysis of Dnm1p

Tobias Rosen

Mitochondria can dynamically change their morphology based on both developmental and physiological cues. These organelles can both fuse and divide to yield long tubular structures or multiple, smaller mitochondria, respectively. Mitochondrial fission is of particular interest because it likely involves the coordinated action of a large protein complex to perform mechanical work. One protein necessary for mitochondrial fission is Dnm1p in yeast (Drp1 in mammals). This molecule has significant homology to dynamin, a heavily-studied protein involved in the membrane fission associated with endocytosis. At a gross structural level, both molecules share a nearly identical predicted architecture that features a N-terminal GTPase domain, a middle or assembly domain, and a C-terminal a-helical GTPase Effector Domain (GED). However, it is unclear how GTP hydrolysis leads to membrane scission in either dynamin or Dnm1p. In fact, there is even controversy over whether these GTPases use GTP hydrolysis to mechanically sever the membrane or act as classic nucleotide-regulated switches.

We are analyzing the biochemical and structural properties of Dnm1p to further characterize its mechanism of action. Using protein dissection, we will examine the interaction of the GTPase and GED domains *in vitro*, which has proven essential in the study of dynamin. So far, through limited proteolysis, we have identified several stable domains, one of which corresponds to the predicted GED region. These fragments are being heterologously expressed and manipulated to characterize the regulation of GTP hydrolysis. To further analyze this interaction, we will attempt to crystallize each of these fragments as well as several full-length orthologs.

245. Examining fusion intermediates of HIV-1 gp41

Takumi Koshiba

Upon binding of gp120 to its cellular receptors, CD4 and certain chemokine co-receptors, gp41 undergoes a multi-step structural transition from a native, nonfusogenic conformation to a fusion-active conformation. Previous work has revealed that the native gp41 converts to a transiently populated "prehairpin" intermediate prior to formation of the fusogenic structure.

To analyze conformational changes in HIV-1 gp41, we have explored the mechanism of action of the HIV-1 entry inhibitor 5-Helix, a designed protein that binds the C-peptide region of gp41 and potently inhibits its fusogenic activity. We constructed several epitope-tagged variants to determine which conformations of gp41 is the target of 5-Helix. We find that binding of 5-Helix to gp41 is strongly activated by interaction of the envelope protein with cellular receptors. These results strongly suggest that the gp41 prefusogenic intermediate is the target of 5-Helix, and that this intermediate has a remarkably "open" structure, with exposed C-peptide regions. These results provide important structural information about this intermediate that should facilitate the development of HIV-1 entry inhibitors and may lead to new vaccine strategies.

246. Analysis of HIV-1 gp41 mutants

Tara Suntoke

Our research is designed to further elucidate the mechanism by which gp41 mediates membrane fusion. The fusion-active 6-helix structure is stabilized predominantly by hydrophobic interactions between N and C helices. Using available structural data for the fusion-active core, we will create a series of mutations that are designed to reduce or prevent N-C association and thus 6-helix bundle formation. A range of hydrophobic

substitutions at each position will be used to test the hypothesis that hairpin formation directly correlates to fusion activity. Stability of the gp41 mutants will be determined biophysically using a 6-helix peptide-based model. This data will be correlated to biological activity in a cell-based system. The fusogenic potential of the gp41mutants expressed in mammalian cells will be analyzed using a cell-cell fusion assay and the presence of conformational changes along the fusion pathway will be determined using conformation-specific antibodies and peptide probes. Finally, since it is unclear whether hemifusion occurs pre- or post- 6-helix bundle formation, the ability of the gp41 mutants to form this intermediate (in which there is lipid mixing but no content mixing) will be tested using a dye-transfer fusion assay.

Associate Professor: Stephen L. Mayo
Research Fellows: Joseph J. Plecs, Julia M. Shifman
Graduate Students: Oscar Alvizo, Daniel N. Bolon, Eun-jung Choi, Deepshikha Datta, Geoffrey Hom, Possu Huang, Kirsten S. Lassila, Jessica Mao, Shannon A. Marshall, Premal S. Shah, Pavel Strop, Christopher A. Voigt
NMR Spectroscopist: Scott A. Ross
Research and Laboratory Staff: Marie L. Ary, Cynthia L. Carlson, Rhonda K. Digiusto

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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.

247. Catalytic protein design

Daniel N. Bolon[1], Stephen L. Mayo

A prominent goal of protein design is the generation of proteins with novel functions, including the catalytic rate enhancement of chemical reactions at which natural enzymes are extremely efficient. The ability to design an enzyme to perform a given chemical reaction has considerable practical application for industry and medicine. Significant progress has been made at enhancing the catalytic properties of existing enzymes; however, the design of proteins with novel catalytic properties has met with relatively limited success. We have developed and implemented a general computational approach for the design of enzyme-like proteins with novel catalytic activities.

Our design approach utilizes a "compute and build" strategy that is based on the physical/chemical principles governing protein stability and catalytic mechanism. For an initial test, we used the catalytically inert 108-residue *E. coli* thioredoxin as a scaffold, the histidine-mediated nucleophilic hydrolysis of *p*-nitrophenyl acetate (PNPA) as a model reaction, and the ORBIT protein design software to compute sequences (Bolon and Mayo, 2001). Ranking of designed catalytic models based upon substrate recognition and active site accessibility identified two promising catalytic positions and surrounding active site mutations required for substrate binding. Experimentally, both candidate protozymes demonstrated catalytic activity significantly above background. One of the proteins, PZD2, displayed "burst" phase kinetics at high substrate concentrations, consistent with the formation of a stable enzyme intermediate. The kinetic parameters of PZD2 are comparable to early catalytic antibodies. But, unlike catalytic antibody design, our design procedure is independent of fold, suggesting a possible mechanism for examining the relationship between a protein fold and its adaptive potential.

In order to begin to explore the fold/function relationship, we are modeling PNPA esterase activity into a new protein scaffold, T4 lysozyme. In addition to exploring fold/function relationships, the ease of obtaining structural information in the lysozyme system should allow us to critically assess our design methods.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

Reference

Bolon, D.N. and Mayo, S.L. (2001) *Proc. Natl. Acad. Sci.* **98**:14274-14279.

248. The importance of polar residues in protein cores

Daniel N. Bolon[1], Stephen L. Mayo

Most globular proteins contain a core of hydrophobic residues that are inaccessible to solvent in the folded state. In general, polar residues in the core are thermodynamically unfavorable except when they are able to form intramolecular hydrogen bonds. Compared to hydrophobic interactions, polar interactions are more directional in character and may aid in fold specificity. In a survey of 263 globular protein structures, we found a strong positive correlation between protein size and the number of polar residues at core positions. In order to probe the importance of buried polar residues, we experimentally tested the effects of hydrophobic mutations at the five polar core residues in *E. coli* thioredoxin, using our ORBIT protein design software to predict the optimal hydrophobic mutations. The combined five-fold mutant protein (IAALV) is less stable than wild type and has an unfolding transition that is substantially less cooperative. NMR spectra, as well as amide deuterium exchange, indicate that IAALV is likely sampling a number of low energy structures in the folded state, suggesting that polar residues in the core are important for specifying a well-folded native structure (Bolon and Mayo, 2001).

In order to further understand the role of polar amino acids at core positions, we performed a detailed hydrogen bond analysis on a set of high-resolution crystal structures. We find that 54% of polar amino acids at core positions form hydrogen bonds to main chain atoms whose hydrogen bond potential is otherwise unsatisfied. Based on the results of this survey, we developed a prudent method for including polar amino acids in core design (Bolon and Mayo, submitted). As an experimental test, we used the prudent polar method to design the thioredoxin core (with the exception of the disulfide bonded cysteine). As a control, we performed the same design using a hydrophobic restriction. Both designs were stabilized relative to wild-type thioredoxin, and the prudent polar design was 2.2 kcal/mol more stable than the

hydrophobic design. These results demonstrate the thermodynamic importance of polar amino acids at core positions.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

References

Bolon, D.N. and Mayo, S.L. (2001) *Biochemistry* **40**:100047-100053.

Bolon, D.N. and Mayo, S.L. (2002) Submitted for publication.

249. Designing proteins with prolines

Eun-jung Choi, Stephen L. Mayo

Recently, there have been many reports of increased protein stability due to single and multiple proline mutations. Proline therefore appears to be an important residue to consider in protein design. Proline residues decrease the conformational entropy of the unfolded state and can be stabilizing when placed at locations where it does not perturb the interactions of other residues in the folded state. Careful selection of proline tolerable mutation sites should therefore be included in a good protein design strategy. However, prolines have been excluded from consideration in our protein designs because our potential function does not include a conformational entropy term. We are using the β1 domain of Streptococcal protein G as a model system to determine how to incorporate proline residues into our designs. We constructed 11 single and double proline mutants and studied their stabilities. As reported previously, mutations within secondary structures are destabilizing, and those at the N-terminus of the α-helix have no effect. However, mutations in loop regions that are exposed and thus make few interactions with other residues, stabilize the protein substantially ($\Delta\Delta G$ = 0.6 kcal/mol). These experiments demonstrate the potential of enhancing protein stability by incorporating proline residues. But they also show that in many cases the favorable entropic benefit is canceled out by unfavorable enthalpic effects.

250. An experimental and computational approach for designing a conformation switch in proteins

Deepshikha Datta[1], Stephen L. Mayo

Our present focus is on combining methods from mean-field theory (derived from statistical mechanics) and computational protein design to understand protein sequence space and eventually design a protein sequence capable of switching between two completely different folds—from a β-sheet to an α-helix. Several roles have been suggested for switch sequences. They may act as evolutionary bridges that serve as intermediates in the pathway for the evolution of new folds. They could be evolutionary end points in the development of allosteric systems. Or they could simply be hazardous dead ends, as in protein misfolding diseases. An important determinant for switching conformation in proteins/peptides is the external environment that induces the switch. We are attempting to achieve a switch by using metal binding as an external force to direct the sequence towards the α-helical conformation over the β-sheet conformation. In designing an amino acid sequence capable of adopting both conformations, we used a strategy of first determining positions on both proteins (protein G and engrailed homeodomain) that are highly tolerant to substitutions to common residues. A mean-field approach was applied to identify the conserved and mutable amino acids on the two proteins. In the next step, the mutable positions are designed using a protein design algorithm to select for common amino acids and consequently, to bring the two sequences closer in sequence space. As the two sequences are brought closer together, the computational results are verified experimentally by characterizing the designed proteins using circular dichroism and NMR.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

251. Full-sequence design and the algorithms behind it

Geoffrey Hom[1], Stephen L. Mayo

The computational full-sequence design (FSD) of a protein is a rarity. To date, only two verified FSDs (both from this lab) have been reported in the literature. Both utilized so-called "exact" algorithms that are precise but may be prohibitively time-consuming. One of the FSDs, a 51-amino acid fragment of the engrailed homeodomain (EH) fold, was done in a step-wise manner. We have used an approximate algorithm, a Monte Carlo simulation coupled with simulated annealing, to do a concerted FSD of the above EH fragment. As in previously reported FSDs, the goal was to optimize stability as opposed to function. The algorithm examined ~10^9 rotameric sequences in 14 CPU hours, and the lowest-energy sequence obtained from the simulation was constructed and expressed in *E. coli*. The resulting protein, EH_FSD, expresses well and is soluble. Spectra from 1D ^{1}H-NMR experiments indicate it is well folded and α-helical. EH_FSD unfolds cooperatively in guanidinium chloride and appears to have a thermal denaturation temperature of at least 99 C, compared to 49 Cfor the wild type. We are currently attempting to determine its crystal structure.

The above results not only help validate our lab's current force field, but also suggest that approximate algorithms can be sufficient for designing entire proteins. However, an exact algorithm is still a useful control for evaluating a force field's accuracy: A negative result obtained using an approximate algorithm may be due to either the force field or the algorithm itself. Accordingly, we are also attempting to improve our lab's current exact algorithms.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

252. Protein-protein recognition

Possu Huang[1], Stephen L. Mayo

Biologically functional proteins carry out their actions by interacting with other components in the cell, and protein-protein association serves a very important role. Proteins can bind directly to their targets to carry out a function or they can bind specifically to themselves forming higher-order structures to perform their duties. We are interested in learning how proteins can utilize their

surface residues to interact with other proteins. We are also curious about the influence protein backbone geometry has on complex formation. A special fast fourier transform (FFT) based docking algorithm was developed to study dimer formation. We can reproduce certain wild-type dimer geometries based on a monomer backbone and we can also generate *de novo* dimers based on protein surface complementarities. The protein design algorithm developed in the Mayo group is being used in conjunction with the FFT docking algorithm to redesign the binding interface. We intend to capture the physics and chemistry in the protein-protein recognition process by simulating the process on computers. Redesigned protein dimers are being synthesized and tested. Their binding specificities and affinities are being assessed by NMR and analytical ultracentrifugation methods.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

253. Computational design of a novel enzyme for catalysis of a pericyclic reaction

Kirsten S. Lassila[1], Stephen L. Mayo

A goal of computational protein design is to design completely new catalytic sites on previously inactive protein scaffolds. Progress toward *de novo* enzyme design can be expected to benefit synthetic chemistry, environmental monitoring and bioremediation, and industrial applications such as detergent and food production. Our laboratory recently demonstrated that computational design could be used to create a new esterase catalytic activity on a thioredoxin scaffold. We hope to expand upon this work by using similar techniques to develop a new protein catalyst for a pericyclic reaction.

We have taken initial steps toward the design of an enzyme that will catalyze the rearrangement of chorismate to prephenate. This Claisen rearrangement has been extensively studied, both as a rare enzyme-catalyzed pericyclic process and as an essential reaction in the biosynthesis of aromatic compounds. By using computational protein design methods to identify protein sequences likely to bind an *ab initio* transition state structure of this reaction, we hope to design a novel catalyst for the chorismate-prephenate rearrangement.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

254. Electrostatic models for protein design calculations

Shannon A. Marshall[1], Stephen L. Mayo

Computational protein design algorithms have typically used fast methods based on Coulomb's law and/or geometry dependent hydrogen bond terms to model electrostatic interactions. Polar desolvation effects have either been neglected in design calculations or approximated using surface area-based or per-atom desolvation penalties. Continuum models of electrostatics such as those based on the finite difference Poisson-Boltzmann method (FDPB) have substantially better predictive power. However, since they are intractable for problems with high combinatorial complexity, FDPB models have not been used to model electrostatics in protein design calculations.

The results of a previous protein design study indicate that the balance among the electrostatic components of the current ORBIT (optimization of rotamers by iterative techniques) protein design force field requires optimization. We assessed the accuracy of the electrostatic terms in the ORBIT force field by comparing its predictions to energies calculated using the finite difference Poisson-Boltzmann method (FDPB). Next, we developed and parametrized two classes of electrostatic models for use in protein design calculations. We identified optimal electrostatic parameters for the ORBIT force field by maximizing the agreement between ORBIT and FDPB energies. The new parameters include surface area-based and Laziridis-Karplus model solvation parameters for polar functional groups and dielectric values for different classes of electrostatic interactions. The optimized parameters dramatically increase the accuracy of the electrostatic energies calculated using a protein design force field while maintaining excellent computational efficiency.

To obtain even greater accuracy, we have developed one- and two-body decomposable formulations of the FDPB model. The new methods produce energies that are very similar to the results of traditional FDPB calculations and are compatible with the computational demands of protein design calculations. These new electrostatic models should significantly aid in efforts to design proteins with desired thermodynamic and functional properties.

[1]*Division of Chemistry and Chemical Engineering, Caltech*

255. Computational design of a novel aldolase

Jessica Mao[1], Stephen L. Mayo

Designed enzymes are attractive industrially for their efficiency, substrate specificity, and stereoselectivity. To date, there are few enzymes used in organic synthesis. The aldol condensation is the chemical reaction between two aldehyde/ketone groups, yielding a β-hydroxy-aldehyde/ketone that upon dehydration by acid or base affords an enone. It is one of the most important and utilized carbon-carbon bond-forming reactions in synthetic chemistry. While natural aldolases are efficient, they are very limited in their substrate range. Novel aldolases that catalyze reactions between desired substrates would be a powerful synthetic tool. They would expand the limited repertoire of designed enzymes and further our understanding of their structure/function relationship. We are adopting the "compute and build" design cycle (Bolon and Mayo, 2001) that combines theory, computation, and experiment to rationally design a novel aldolase. Our initial design will catalyze the reaction between acetone and benzaldehyde via the enamine mechanism. The backbone structure of triosephosphate isomerase (TIM) will serve as the scaffold for building a novel protein catalyst. Potential catalytic sites are being identified using the ORBIT protein design software and modeling a high-energy state in the reaction mechanism as a series of rotamers. The validity of this approach was indicated by identification of the natural catalytic lysine of catalytic

antibody 33F12, which catalyzes the target reaction. pK_a calculations are being performed to validate the designed sequences, and experimental validation will soon be underway.

[1]*Division of Chemistry and Chemical Engineering, Caltech*

Reference

Bolon, D.N. and Mayo, S.L. (2001) *Proc. Natl. Acad. Sci. USA* **98**:14274-14279.

256. Torsional mechanics using multiple-torsion degrees of freedom

Joseph J. Plecs, Stephen L. Mayo

We have developed a method for constructing multiple-torsion degrees of freedom for a protein main chain. These degrees of freedom retain the efficiency of torsional coordinates, while avoiding many of the disadvantages of standard torsions. Compared to Cartesian (xyz) coordinates, torsional or dihedral coordinates sharply reduce the conformational space to be explored, and torsions can achieve improved results in both radius of convergence and the speed of the calculation in simulated-annealing searches. Torsional coordinates can also permit the use of longer timesteps in dynamics calculations by eliminating the high-frequency oscillations associated with distortions in covalent geometry. The value of torsions for molecular calculations has been limited, however, by several problems. In the case of long chains, such as the protein backbone, torsional energy gradients can be dominated by the atoms with the longest moment arm with respect to the axis of rotation—generally, the atoms farthest from the angle being varied. It is unrealistic that rotations about a particular angle should be dominated by the interactions farthest away, because in practice the flexibility of the chain will dissipate the effects of faraway interactions. These faraway interactions also require the use of relatively small steps in torsional dynamics or minimization, because the energy gradients associated with them can become very steep. Another limitation is that closed loops such as cyclic peptides cannot be handled at all by single-torsion methods without allowing distortions of covalent geometry. We resolve these problems by constructing new systems of coordinates: Combinations of main-chain torsions that permit us to adjust one region of the main chain without distorting the local covalent geometry and without disturbing the main chain outside the local region.

257. Structures of computationally designed proteins

Scott A. Ross, Shannon A. Marshall[1], Premal S. Shah[2], Stephen L. Mayo

Demonstrating experimentally that designed proteins adopt their target folds is an important validation of the protein design algorithm. We are presently working to solve the structures of two designed variants of engrailed homeodomain by NMR. Due to low sequence diversity in the designed proteins, it has proven necessary to use triple resonance 3D and 4D NMR methods despite the small size of the proteins (51 residues). Sequential assignment has been completed for one variant and initial trial structures have been generated. The preliminary data indicate that this protein does indeed adopt a fold similar to that of the native protein. Data collection and sequential assignment are underway for the second variant.

These variants differ substantially in sequence from the native protein (24% homology) and from each other (45% homology). If indeed both prove to have the homeodomain tertiary structure, it will demonstrate the broad range of sequence space spanned by the fold, as well as validate the calculations. Furthermore, both variants are full sequence designs. To date, only one structure of a fully computationally-designed protein has been reported, a 27-residue $\beta\beta\alpha$ fold from our lab. We are very interested to expand the database to see whether common structural features emerge peculiar to our designed proteins, or whether they are indistinguishable from native proteins.

[1]*Division of Chemistry and Chemical Engineering, Caltech*

[2]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

258. Force-field improvement and optimization techniques in protein design

Premal S. Shah[1], Stephen L. Mayo

The effectiveness of protein design hinges on two components: (1) a force field that accurately describes the physical interactions within proteins, and (2) an efficient search algorithm that rapidly searches sequence space to identify the minimal energy solution. Our recent work has focused on both of these elements.

One component of our force field is a hydrophobic solvation term that is based on exposed and buried side-chain surface areas. Current methods involve a pairwise approximation of surface areas between pairs of interacting rotamers, which are statistically significant conformations of amino acids. Inaccurate surface areas are often a problem when three or more rotamers interact. We developed a method for calculating an exact surface area for rotamers. Using a Monte Carlo (MC)-based approach to approximate the global minimum energy conformation (GMEC), a rotamer's surface area can be modeled and calculated exactly within a low energy solution. The validity of this approach was confirmed experimentally; proteins predicted with this method had significantly higher stabilities than those obtained using the previous approach.

We also developed a novel search algorithm that pre-processes rotamers prior to evaluation with exact algorithms such as dead-end elimination (DEE). If DEE algorithms converge, the solution is guaranteed to be the GMEC. However, they often fail for large problems. Our new algorithm, termed Vegas, samples rotamers in low-energy solutions using approximate search algorithms such as MC and self-consistent mean-field (SCMF). Rotamers whose approximate solutions have relatively high energies are eliminated from the calculation. Using this method, we observed significant computational cost reductions compared to using DEE alone. We have also been able to obtain solutions to DEE-intractable problems that have lower energies than those obtained using any previous method.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

259. Computational design of calmodulin for improved binding specificity

Julia M. Shifman, Stephen L. Mayo

We are using computational methods to redesign calmodulin (CaM) to bind tightly to only one of its targets, smooth muscle myosin light chain kinase (smMLCK), increasing CaM's binding specificity. The CaM binding interface is optimized using ORBIT (optimization of rotamers by iterative techniques), a protein design program that uses a physically based force field and the Dead-End Elimination theorem to compute sequences that are optimal for a given protein scaffold. However, ORBIT does not accurately represent electrostatic interactions across the CaM-smMLCK binding interface. We addressed this problem during the last year. We modified our sequence optimization procedure to emphasize interactions across the CaM-target binding interface by scaling up intermolecular terms in the energy expression. We also explored different values for the dielectric constant, the size of the rotamer library, and the number of optimizable positions. These calculation parameters were evaluated by constructing the predicted CaM molecules and determining their binding affinities to a set of peptide targets. The best CaM variant predicted with the new method retained the wild-type binding affinity to the main target, smMLCK. Its binding specificity for smMLCK was increased compared to both wild-type and a previously reported core variant, CaM_8. The new CaM variant also provided an especially remarkable discrimination against skMLCK, the target whose sequence is most similar to the desired target, smMLCK. These results suggest that continued efforts will produce a CaM variant with high binding specificity in the near future. In addition, our modifications to the force field helped us to simulate binding interaction energies more accurately.

260. Structure of a 1:1 polyamide-DNA complex

Adam R. Urbach[1], John J. Love[2], Scott A. Ross, Peter B. Dervan[3]

The Dervan lab has done a great deal of work designing small molecules that bind to DNA with high affinity and sequence specificity. These polyamide ligands bind to the minor groove of DNA in 1:1 and 2:1 ligand to DNA stoichiometries. While the structural basis of 2:1 binding had been relatively well characterized, that of 1:1 binding had not. We therefore set out on a collaboration to determine the solution structure by 2D ^{1}H NMR methods of a polyamide-DNA complex known to display 1:1 stoichiometry (Urbach *et al.*, 2002).

NMR spectra of the complex were remarkably well-dispersed, facilitating sequential assignment and providing an abundance of NOE-derived distance restraints. The NMR data showed the expected 1:1 stoichiometry and unique orientation. The ligand lies deep in the minor groove of the DNA for a full turn of the helix. The final ensemble was of sufficiently high precision to reveal atomic details of the structural basis of 1:1 complexation. The ligand and DNA are found to be in register such that each of the ligand's amide NH groups forms a bifurcated hydrogen bond to base pair atoms in the floor of the minor groove. Other favorable interactions were observed which suggest a structural basis for the sequence selectivity and orientation preference. This work provides a foundation for further refinement of sequence-specific ligands for DNA.

[1]*Department of Chemistry, Harvard University, Cambridge, MA*
[2]*Department of Chemistry, San Diego State University, San Diego, CA*
[3]*Division of Chemistry and Chemical Engineering, Caltech*

Reference

Urbach, A.R., Love, J.J., Ross, S.A. and Dervan, P.B. (2002) *J. Mol. Biol*. **320**:55-71.

261. Computationally optimizing *in vitro* protein evolution

Christopher A. Voigt[1], Zhen-Gang Wang[2], Frances H. Arnold[2], Stephen L. Mayo

We are developing new strategies in the design of proteins by combining two powerful but largely separate methodologies: that of rational computational design and that of directed evolution. This research has two primary focuses: (1) to identify candidate residues for saturation mutagenesis, and (2) to predict the optimal crossover locations and the best sets of parents for recombination. We first use simple models, based on statistical mechanics, to study the gross dynamics of the evolution algorithm. We then use principles gleaned from the simple models to guide computational structure design tools to rapidly eliminate sequences that would destabilize the three-dimensional structure of a specific protein. As an example of this strategy, I will describe our computational and experimental work to optimize *in vitro* sexual recombination. Theory developed to understand genetic algorithms hypothesizes that the optimal crossovers are those that least disrupt structural building blocks. Based on this assumption, we have developed a computational method to predict the optimal crossover locations and demonstrate that the crossovers observed in functional hybrid proteins correlate with these predictions. Collectively, these algorithms enable the exploitation of the rapidly growing database of protein structures and sequences in developing improved enzymes for industrial and pharmaceutical applications.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*
[2]*Division of Chemistry and Chemical Engineering, Caltech*

262. Improving protein design calculations

Eric Zollars[1], Stephen L. Mayo

My projects since entering the Mayo lab have involved the development of our computational protein design package. One of the major assumptions that allows protein design to occur is the assignment of amino acid side chains to discrete conformations known as rotamers. Without this discretization, the complexity of computational protein design would be astronomical.

However, rotamer usage leads to error in side chain placement and consequently, errors in the predicted sequence and/or energy of the designed protein. An optimal rotamer library would contain the minimum number of rotamers that would still yield reasonably accurate results. The current rotamer library in use in the Mayo lab is derived from a published statistical survey of the conformations of side chains observed in protein crystal structures. Research by other labs since that published survey have shown inherent problems with this approach. Two new approaches were put forward: a Bayesian analysis of the structural database to compensate for areas of conformational space that were inadequate in producing statistical significance, and a study that included energy considerations in developing the conformations of the discrete rotamers. We implemented these improved rotamer libraries for use in our protein design algorithms, used them to design sequence variants of protein G, and built the designed variants in the lab. Thermodynamic characterization of the variants was then performed to evaluate the utility of these new rotamer libraries.

A burgeoning area of research in protein design is the development and implementation of better models to approximate the electrostatic interactions that occur in proteins in solution. We are in the process of validating the efficacy of a variety of these models for use in protein design calculations.

[1]*Graduate Option in Biochemistry and Molecular Biophysics, Caltech*

Publications

Bolon, D.N. and Mayo, S.L. (2001) Enzyme-like proteins by computational design. *Proc. Natl. Acad. Sci. USA* **98**:14274-14279.

Bolon, D.N. and Mayo, S.L. (2001) Polar residues in the protein core of *E. coli* thioredoxin are important for fold specificity. *Biochemistry* **40**:100047-100053.

Bolon, D.N., Voigt, C.A. and Mayo, S.L. (2002) *De novo* design of biocatalysts. *Curr. Opin. Chem. Biol.* **6**:125-129.

Datta, D., Wang, P., Carrico, I.S., Mayo, S.L. and Tirrell, D.A. (2002) A designed phenylalanyl-tRNA synthetase variant allows efficient *in vivo* incorporation of aryl ketone functionality into proteins. *J. Am. Chem. Soc.* **124**:5652-5653.

Gordon, D.B., Hom, G.K., Mayo, S.L. and Pierce, N.A. (2002) Exact rotamer optimization for protein design. *J. Comp. Chem*. In press.

Lassila, K.S., Datta, D. and Mayo, S.L. (2002) Evaluation of the energetic contribution of an ionic network to β-sheet stability. *Protein Sci.* **11**:688-690.

Marshall, S.A., Morgan, C.S. and Mayo, S.L. (2002) Electrostatics significantly affect the stability of designed homeodomain variants. *J. Mol. Biol.* **316**:189-199.

Shifman, J.M. and Mayo, S.L. (2002) Modulating calmodulin binding specificity through computational protein design. *J. Mol. Biol.* In press.

Voigt, C.A., Mayo, S.L., Arnold, F.H. and Wang, Z. (2001) Computationally focusing the directed evolution of proteins. *J. Cell Biochem. Suppl.* (37):58-63.

Voigt, C.A., Martinez, C., Wang, Z.G., Mayo, S.L. and Arnold, F.H. (2002) Protein building blocks preserved by recombination. *Nat. Struct. Biol.* **9**:553-558.

Voigt, C.A., Mayo, S.L., Wang, Z-G. and Arnold, F.H. (2002) Directing the evolvable: Utilizing robustness in *in vitro* evolution. In: "Robustness," Erica Jen, ed., Santa Fe Institute Press, Santa Fe, New Mexico. In press.

Ethel Wilson and Robert Bowles Professor of Cell Biology: James H. Strauss
Senior Research Associate: Ellen G. Strauss
Postdoctoral Scholar: Pritsana Chomchan, Jeroen Corver
Research Laboratory Staff: Maria Farkas, Edith Lenches, Kayla Smith
Visitor: Ellen Marion Westerhout[1]
[1]*Leiden University, The Netherlands*

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Summary: We study two groups of animal viruses, the alphaviruses and the flaviviruses. These viruses contain an RNA genome of 10-12,000 nucleotides and are enveloped, having a lipid envelope that surrounds an icosahedral nucleocapsid. Both alphaviruses and flaviviruses are vectored by blood-sucking arthropods (they are arboviruses) and can infect a wide range of arthropods as well as vertebrates. We wish to understand the replication of alphaviruses and flaviviruses at a molecular level and to study the evolution of these two virus groups and more broadly the evolution of all RNA viruses.

There are more than 26 recognized alphaviruses and more than 70 known flaviviruses. Many of these viruses are important human pathogens, causing hundreds of millions of cases of human disease annually. Diseases caused by the alphaviruses include encephalitis, polyarthritis, and febrile illness accompanied by rash, and flaviviral diseases include hemorrhagic fever and encephalitis as well as febrile illness accompanied by rash. West Nile virus, which appeared three years ago for the first time in North America and has now spread throughout the United States, is a member of this family.

The genome RNAs of both virus groups are infectious and are translated into long polyproteins that are cleaved to give nonstructural proteins required for RNA replication. In flaviviruses, the genome is the only mRNA and the polyprotein also contains structural proteins required for virus assembly. In alphaviruses, a subgenomic mRNA is produced that is translated into the structural proteins.

Our studies have been greatly aided by the development of cDNA clones that allow us to express all or parts of the viral genome. In the case of alphaviruses we have constructed complete cDNA clones of several viruses, including Sindbis, Ross River, and Semliki Forest viruses, from which infectious virus can be recovered after transcription *in vitro* with T7 or SP6 polymerase and transfection of susceptible cells. Similar full-length clones have been derived for the flaviviruses, but they are in general unstable and possess "poison sequences" that limit their ability to replicate. However, we have made extensive use of a full-length clone of yellow fever 17D virus and are constructing a full-length clone of dengue 2 (DEN2) virus.

Recently, we have devoted considerable effort towards producing flavivirus preparations of sufficient purity and homogeneity for examination by cryoelectron microscopy. In collaboration with researchers at Purdue University, we have obtained a structure for dengue 2 (DENS) virus at about 20 Å resolution, and are now analyzing immature dengue virions that contain uncleaved prM. These studies, which comprise the first reconstructions of a flavivirus, have shown dengue to possess a unique structure with elements of icosahedral symmetry, but in which the protein subunits are not all in quasi-equivalent positions. We are using this structure as a basis for probing the function of the viral envelope proteins by site-specific mutagenesis.

263. Construction of a full-length dengue 2 clone

Jeroen Corver, Pritsana Chomchan

Dengue and dengue haemorrhagic fever have emerged as a major public health problem. An estimated 50 million dengue infections occur annually and the geographical spread, incidence, and severity of dengue fever and dengue haemorrhagic fever epidemics are increasing in the tropics. Development of safe, effective and inexpensive vaccines for dengue (DEN) will be essential to control and prevent spread of this disease. Development of a full-length infectious cDNA clone of dengue is an important first step towards constructing mutant viruses for use as potential vaccines and for studying viral pathogenesis, structure, and immunity.

Our laboratory studies a candidate vaccine strain of DEN2 virus (S1 strain, derived from the PR-159 isolate), and we want to generate a full-length clone of this strain. RNA was isolated from purified DEN2 virus grown on C6/36 mosquito cells. After synthesis of cDNA by reverse transcriptase, the complete sequence was cloned into six separate fragments. Sequencing revealed no mutations, as compared to our previously published sequence of the virus. Since flavivirus sequences, and those of DEN virus in particular, are known to be toxic and instable in bacteria, we decided to construct the full-length clone in pBeloBac11, a plasmid with a copy number of 1. In the past we have successfully used the same vector to make another full-length flavivirus clone, pBAC/FLYF, encoding yellow fever virus. Four of the six fragments of the DEN genome have been subcloned into pBeloBac11. Using our partial clones, the Purdue group assembled a full-length DEN clone, but discovered that it was not infectious, due presumably to errors introduced during PCR amplification. We have begun swapping fragments from our partial clone into their (dead) full-length clone.

264. Construction of a chimeric virus with the YFV backbone and capsid, prM, and E of DEN2

Edith Lenches, Pritsana Chomchan

In order to study the interaction between the structural proteins of a flavivirus with one another and with the replication machinery, we set out to construct a chimeric virus in which nucleotides encoding the three structural proteins of dengue virus were substituted for sequences encoding their counterparts in a full-length infectious cDNA clone of yellow fever virus. We had already determined that the construct in which the DEN prM and E moieties had replaced those of yellow fever grew almost as well as the wt YFV. Using this chimera as

a starting place, we wanted to insert the dengue C protein coding sequences as well.

Our laboratory strain of dengue virus type 2 is a vaccine derivative from a Puerto Rican isolate, PR-159 S1, and our laboratory had previously constructed a partial cDNA clone containing the structural protein genes (see Abstract #263. The sequence for the capsid protein was amplified from our DEN2 cDNA clone by PCR, and the PCR products ligated into our yellow fever cDNA backbone. The yellow fever clone was obtained from the laboratory of Charles Rice, and is present in a high copy number plasmid vector. RNA was transcribed *in vitro* from the chimeric clone and transfected onto both BHK-21 (vertebrate) and C6/36 (mosquito) cells. The RNA transfection on BHK-21 and C6/36 was very poor, and there was no spreading, and no detectable virus released, even after passing the supernatant from the transfected cells again on both cell lines. This indicates that the capsid protein contains sequences essential for replication that apparently cannot be supplied to the yellow fever replicase by the heterologous dengue capsid protein. The reason for this is presumably incompatibility in the cyclization sequence such that the dengue capsid cannot interact successfully with the yellow fever cyclization sequence at the 3' end of the genome. Mutational studies are underway to change residues in the YF 3' NTR to match the dengue sequence and conversely to mutate the dengue capsid sequence to match the YF 3'NTR.

265. Production of immature YFV containing prM for cryoelectron microscopy

Edith Lenches, Pritsana Chomchan

During assembly of the flavivirus, yellow fever virus, the genomic RNA associates with the capsid protein to form a nucleocapsid or core, while the two transmembrane glycoproteins of the virus, prM and E, interact with one other in the membrane of the endoplasmic reticulum to form heterodimers. When the nucleocapsid buds into the lumen of the ER, it acquires a lipid envelope containing prM and E. These "immature" virus particles containing prM are transported to the cell surface through the Golgi apparatus. The trans-Golgi compartments are known to be mildly acid, and this acid treatment produces a conformational change in the prM protein, rendering it cleavable by furin, a host cell Golgi protease. As a final step in the maturation of the infectious particle, prM is cleaved by furin to the "pr" portion, which is lost from the particle, leaving the membrane-associated M protein of 75 amino acids. Since the structure of the mature particles, as determined by cryoelectron microscopy, has been shown to be unusual,[1] we wanted to compare it to the structure of the immature particles. For other flaviviruses, particles produced in insect cell culture contain large amounts of (uncleaved) prM, but our preparations of mosquito-cell grown flavivirus contain almost no prM. To obtain immature particles, we added 20 mM ammonium chloride to the cell culture medium, which raises the pH of the trans Golgi compartment, preventing the furin cleavage.

BHK-21 cells were infected with our YFV glycosylation mutant (see Abstract #268) at 1 pfu/cell and incubated for 48 hr at 37°C in the presence of 20 mM ammonium chloride. The medium containing the released virus was harvested, and precipitated with 8% PEG (polyethylene glycol, avg. mol. weight 8000) and resuspended in buffer. We could concentrate the virus further by a second precipitation with 4% PEG. Since yellow fever virus is unstable in both sucrose and tartrate gradients, we are still looking for a suitable gradient in which to purify the immature particles.

Reference

[1]Kuhn, R.J. *et al.* (2002) *Cell* **108**:717-725.

266. *In vitro* reactivation of immature dengue virus particles

Pritsana Chomchan, Jeroen Corver

As described in the previous abstract, by addition of 20 mM ammonium chloride to the cell medium, which neutralizes cellular low-pH compartments, it is possible to produce immature flavivirus particles containing uncleaved prM. When such purified prM–containing immature dengue particles were examined by cryoelectron microscopy, the reconstruction showed particles with "spikes" protruding from their surface, in contrast to mature dengue virions which appear quite smooth in comparable reconstructions. This reconstruction was described in last year's Annual Report (Abstract #289).

The yield of immature particles was initially disappointingly low, although it is difficult to measure accurately, due to the greatly reduced specific infectivity of prM-containing particles relative to wild-type virions. On the other hand, additional reconstructions to higher resolution will require significant amounts of material. We have been able to improve the production of immature dengue virus by replacing medium on insect cell culture (containing 1% fetal calf serum) 12-24 hrs prior to infection with dengue virus. This modification increased the yield in pfu 10-fold, and assuming that the specific infectivity is not altered, this means that the production of (non-infectious) immature particles is also increased 10-fold. In the case of another flavivirus, tick-borne encephalitis virus, it has been shown that prM-containing particles could be reactivated by exposure to low pH and subsequent treatment with exogenously supplied furin, and that such a reactivation restored infectivity. We are currently attempting similar reactivation of immature dengue type 2 particles, hoping to obtain sufficient reactivated particles for cryoelectron microscopy, to confirm that the cryoelectron structure of prM-containing particles observed is indeed an intermediate in virus assembly and maturation, and not an artifact of preparation. We have purified prM-containing particles, but after treatment of this preparation with low pH and furin, we could not detect a significant increase in viral titer, nor could we see any effect on the prM protein as analyzed by SDS-PAGE. It is clear that the conditions for enzymatic cleavage need to be optimized.

267. Cyclization of the YFV RNA genome

Jeroen Corver, Trisha Sando, R. Aaron Robison, Kayla Smith, Edith Lenches, Pritsana Chomchan

We have cloned a yellow fever virus (YFV) replicon, in which most of the structural genes have been deleted. Only the first twenty amino acids of the capsid protein (the N-terminal part of the initially synthesized polyprotein) was preserved to ensure replication (C20 replicon). Replication was measured by immunofluorescent staining of BHK cells transfected with *in vitro* synthesized replicon RNA. The sequence encoding the first 20 residues of the capsid protein has previously been shown to be involved in replication of the YFV RNA. It was shown that part of that particular sequence is complementary with a region in the 3'NTR, suggesting that these two sequences may interact, thus forming a possible circular RNA intermediate. Others have shown that point mutations in the proposed cyclization sequence of Kunjin virus[1], a related flavivirus, abolishes replication. We were able to reproduce this in YFV. In addition, it was shown that introduction of compensatory mutations in the 3'NTR restored the RNA replication capacity. We deleted parts of the C20 replicon to further investigate the sequence involved in replication. Amino acids 15-20 could be deleted without disturbing the replication competence. In addition, aminoacids 1-8 could be deleted as well, thereby defining the 5'end of the required sequence. So, we clearly narrowed down the mandatory sequence for replication to the sequence encoding amino acids 9-15 of the capsid protein of YFV.

Reference

[1]Khromykh, *et al*. (2001) *J. Virol*. **75**:6719-6728.

268. Glycosylation mutant of yellow fever virus

Kayla Smith, Edith Lenches

Flaviviruses are notorious for growing poorly, and until recently it has not been possible to obtain sufficient quantities of homogenous virus preparations to obtain a cryoelectron microscopic reconstruction. In March 2002 our laboratory, in collaboration with the structure group at Purdue University, published a structure of dengue 2 virus.[1] The structure was solved by uniquely fitting the known X-ray crystallographic structure of the major outer glycoprotein, the E protein, from a related flavivirus, tick-borne encephalitis virus, into the cryoelectron density envelope of dengue.

Although we have been trying for some time, we have been unable to produce yellow fever virions in sufficient quantity and of high enough quality for a comparable analysis, and have been looking for ways to improve the stability of the particles. The E proteins of flaviviruses consist of between 493 and 501 amino acids, and most contain specific motifs (N-X-S/T) which are glycosylated at the N (asparagine) by host cell enzymes. When these sites are glycosylated, they serve as landmarks for structural analysis by computer programs. However, the E protein of yellow fever virus (vaccine strain 17D) does not have any glycosylation sites. By site-specific mutagenesis, we have inserted a glycosylation site into YFV E protein at a glycosylation site conserved in other flaviviruses, changing AAU to ACU (encoding amino acid153 of E which is amino acid 436 of the polyprotein). This mutates an Asn to a Thr, so that the glycosylation site reads N-W-T-T instead of N-W-N-T. Surprisingly, this mutant grew better in BHK-21 tissue culture than the original yellow fever 17D, yielding up to 1 x 10^8pfu per ml. We are preparing large amounts of this material for purification for cryoelectron microscopy. The purification procedure involves precipitation with polyethylene glycol, followed by isopycnic centrifugation in potassium tartrate density gradients, and concentration of the material from the band with Centricon™ tubes.

Publication

[1]Kuhn, R.J. *et al* (2002) *Cell* **108**:717-725.

269. Mutagenesis of the BC-loop of Sindbis E1 glycoprotein

Kayla Smith

It has recently been reported by a European laboratory that the crystal structure of glycoprotein E1 of alphaviruses is remarkably similar to the structure of glycoprotein E of flaviviruses, suggesting that these two proteins may have evolved from a common ancestral protein. It is known that both E1 and E contain sequences for fusion peptides at one end of an extended domain. It has been proposed that these internal fusion peptides may interact with adjacent loop structures in the proteins, perhaps by the formation of a classical "beta-barrel" to promote fusion of the viral and cellular membranes during viral penetration and cell entry. In Sindbis virus, an alphavirus, these loops are held together with two disulfide bridges composed of cysteine residues. Since it is unknown whether these residues have sequence-dependent functions other than bridge formation, we have decided to mutate each of the two cysteines of one bridge (amino acids 62 and 94 from the N-terminus of E1) to both alanine and serine, in order to study the effect of these changes on the fusion phenotype. The mutations were introduced into subclones of Sindbis virus by PCR with mutated primers, and then transferred by standard cloning techniques into a full-length cDNA clone of Sindbis from which infectious RNA can be transcribed *in vitro*. These constructs will then be tested for infectivity after RNA transfection.

270. Purification of Sindbis nsP2 proteins

Maria T. Farkas

We have begun a collaborative project with crystallographers at Purdue University to determine the three-dimensional structures of the alphavirus nonstructural proteins, starting with nsP2. Nonstructural protein nsP2 is a bifunctional protein in which the N-terminal domain contains a helicase involved in RNA replication and transcription and the C-terminal domain contains the protease that is responsible for proteolytic processing of the nonstructural polyprotein, p1234. Determination of the crystal structure of this protein will play a vital role in understanding alphavirus RNA replication and in designing inhibitors of virus replication for use as antivirals.

We have cloned the entire sequence encoding nsP2, as well as that encoding each of the functional

domains separately, into the pET23a bacterial expression vector. In this system the target genes are cloned under the control of T7 promoter and upstream of a six-His tag. BL-21 SI competent cells that contain a chromosomal insertion of the T7 RNA polymerase gene under the control of the proU promoter have been transformed with the nsP2-containing vectors. In these cells, expression of the T7 RNA polymerase and the genes on the plasmid under the control of the T7 promoter is induced by the addition of NaCl (salt-inducible expression). Experimentally we have determined the optimum NaCl concentration for expression, which differs for each protein. It was thought that it would be easier to produce and purify the two domains separately, but ultimately we may need to produce the entire nsP2 as a single protein for crystallographic studies.

At the current time we express 1-1.5 mg of soluble protease (the C-terminal domain) per liter of bacterial culture and this material can be purified by a Ni-column followed by HPLC. Expression at this level makes it feasible to generate sufficient quantities of pure protein for crystallography. Unfortunately in similar experiments with plasmids containing the helicase domain, most of the viral protein is sequestered in insoluble inclusion bodies, and only a small fraction of the protein could be purified on a Ni-column. We are trying to increase the expression of soluble helicase by altering the culture conditions before attempting a large-scale purification. At the same time we are also trying to solubilize the helicase protein from inclusion bodies and renature it after solubilization.

271. Production and purification of Sindbis V/33/50/23 for cryoelectron microscopy

Edith Lenches

Sindbis V33/50/23 is a Sindbis variant selected to be resistant to Mabs 33, 50, and 23, three neutralizing monoclonal antibodies. This triple variant is neutralized only 10% as efficiently as wild-type Sindbis, strain AR339, and has a number of amino acid changes relative to AR339. All variants resistant to Mab33 have changes at Gly132 of E1, in this case to Arg. Resistance to Mab 50 is due to changes at amino acids 190 and 205 of glycoprotein E2. All variants resistant to Mab 23 have changes at Lys 216 of E2, most have Lys216->Glu but this variant has Lys 216-> Asn, creating a new glycosylation site of the form Asn-Leu-Thr at amino acids 216-218. The E2 from this variant has an altered electrophoretic mobility, indicating that this glycosylation site is indeed modified. We hope to use this variant to map E2 in particles by subtraction of cryoelectron microscopic density, as has been done with other alphavirus glycosylation mutants.

BHK-21 cells were infected with a multiplicity of 0.1 pfu/cell and incubated at 37°C for 20 hr. The culture medium containing the virus was harvested, and virions precipitated by addition of polyethylene glycol (avg. mol. weight 8000) to 8% w/vol for 2 hr at 4°C. The precipitate was collected by centrifugation, the pellet was resuspended in buffer, clarified by a brief low speed centrifugation, and layered over onto sucrose gradient. To form the gradient, 2.5 ml each of 40%, 30%, 20%, and 10% sucrose in the same buffer were layered sequentially in tubes for the SW40 rotor and placed at 4°C overnight. Gradients were centrifuged for 1 hr at 32,000 rpm at 4°C. The virus band was collected and concentrated to a final volume of 100µl using Centricon™ tubes. Recovery of PFU in the concentrated virus was 64% of that in the clarified solution after PEG precipitation.

Publication

Corver, J., Lenches, E.M., Smith, K., Robison, R.A., Sando, T. and Strauss, J.H. (2002) Fine-mapping of a *cis*-acting sequence element in yellow fever virus RNA that is required for RNA replication and cyclization. *J. Virol.* In press.

Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S. and Strauss, J.H. (2002) Structure of dengue virus: Implications for flavivirus organization, maturation, and fusion. *Cell* **108**:717-725.

Shirako, Y., Strauss, E.G. and Strauss, J.H. (2002) Modification of the 5' terminus of Sindbis virus genomic RNA allows nsP4RNA polymerases with non-aromatic amino acids at the N terminus to function in RNA replication. *J. Virol*. **76**:10188-10194.

Strauss, J.H. and Strauss, E.G. (2002) *Viruses and Human Diseases*. Academic Press, San Diego, 393 pp.

Strauss, E.G., Lenches, E.M. and Strauss, J.H. (2002) Molecular genetic evidence that the hydrophobic anchors of glycoproteins E2 and E1 interact during assembly of alphaviruses. *J. Virol.* In press.

Zhang, W., Fisher, B.R., Olson, N.H., Strauss, J.H., Kuhn, R.J. and Baker, T.S. (2002) Structure of Aura virus suggests that the T=4 organization is a fundamental property of the viral structural proteins. *J. Virol.* **76**:7239-7246.

Cellular, Molecular and Developmental Neurobiology

David J. Anderson
Seymour Benzer
William J. Dreyer
Mary B. Kennedy
Henry A. Lester
Paul H. Patterson
Erin M. Schuman
Kai Zinn

Professor of Biology: David J. Anderson
Research Fellows: Xinzhong Dong, Limor Gabay, C.J. Han, Jaesang Kim, Walter Lerchner, Sally Lowell, Raymond Mongeau, Yosuke Mukoyama, Kenji Orimoto, Gregory S. Suh, Mark Zylka
Graduate Students: Gloria Choi, Christian Hochstim, Donghun Shin, Qiao Zhou, Mariela Zirlinger
Research and Laboratory Staff: Jung Sook Chang, Elizabeth Chiang, Liching Lo, Gina Mancuso, Gabriele Mosconi, Alex Negrillo, Laurent Van Trigt, Peng Wu, Joana Yamada

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National Institutes of Health

Summary: There are currently three major areas of investigation in this laboratory: the development of neural stem cells; the development of arteries and veins; and the functional neuroanatomy of emotional behaviors.

Our studies of neural stem cells encompass both the central nervous system (CNS) and peripheral nervous system (PNS). The latter derives from the neural crest. We have isolated and characterized neural crest stem cells, and are investigating the control of the differentiation and self-renewal of these cells by both cell-extrinsic and cell-intrinsic factors. Our experimental approaches include *in vitro* clonal analysis, *in vivo* transplantation, and loss- and gain-of-function genetic manipulations in mouse and chick embryos. Subtractive hybridization and microarray analysis are being used to identify genes that distinguish stem and progenitor cells at various stages of development. The function of these genes may be tested by both gain- and loss-of-function manipulations in cultured neural crest stem cells, in the latter case using RNA interference (RNAi). One major new line of investigation concerns the function of a new subclass of basic helix-loop-helix (bHLH) transcription factors, called *Olig* genes, that appear to control both motoneuron and oligodendrocyte differentiation in the CNS.

Our studies of the circulatory system stem from our serendipitous discovery that arteries and veins are genetically distinct from the earliest stages of angiogenesis. Arteries express the transmembrane ligand ephrinB2, and veins express one of its receptors, EphB4. Gene-targeting studies have indicated that reciprocal ephrinB2-EphB4 signaling is essential for proper cardiovascular development, and may mediate bi-directional communication between arteries and veins. Our recent studies have included a further analysis of ephrinB2-EphB4 function in angiogenesis, using cell type-specific gene targeting; and experiments to understand the developmental origins of vessel identity. We have also identified important interactions between the developing nervous system and circulatory system, and are exploring the biological function and molecular mechanism of these interactions.

A recent initiative in the lab has begun to develop and apply molecular biological tools to map and manipulate the neural circuitry underlying emotional behaviors, in mice. These studies currently concern two distinct but related subjective emotional states: pain and fear. In the former case, we have identified a novel family of G protein-coupled receptors (GPCRs) for neuropeptides, which are specifically expressed in restricted subsets of nociceptive sensory neurons. We are using homologous recombination in embryonic stem cells to delete these genes to study their function, as well as to study the function and connectivity of the neurons that express these receptors. In collaboration with Professor Melvin Simon's laboratory, we are also carrying out systematic screens to identify ligands for these receptors. This system defines a platform from which to identify the particular nociceptive modalities mediated by these neurons, and to begin to understand the control of the perception of these noxious stimuli, by tracing their connectivity to the brain. These studies may also lead to the development of novel drugs that can be used to treat currently intractable forms of pain in humans, such as neuropathic (nerve injury-related) pain.

In a separate project, we have developed a novel experimental system to study the neural circuits that control behavioral responses to innately fearful stimuli. We have identified unimodal sensory stimuli that can elicit different fear behaviors (flight or freezing) in laboratory mice, depending on the context and/or prior experience of the animals. We are using quantitative immediate early gene expression analysis to map, with single-cell resolution, the brain regions that are active in these different behaviors. Efforts to identify genes expressed in these regions, both constitutively and in an activity-dependent manner, are also underway, using oligonucleotide microarrays and laser-capture microdissection. Identification of molecular markers for brain regions associated with emotional behaviors should permit us to determine the function of these regions, by creating transgenic animals in which the activity of neurons in these regions can be reversibly silenced (using techniques being developed in Professor Henry Lester's laboratory), as well as to map their connectivity. These studies should better define the neural substrates of subjective emotional states, and ultimately may aid in a clearer understanding of the pathophysiological basis of affective disorders in humans, such as anxiety and depression.

272. Transient expression of ngn-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate

Mariela Zirlinger, Liching Lo, David J. Anderson

Lineage-tracing experiments have indicated that some pre-migratory neural crest cells (NCCs) are pleuripotent, generating sensory and sympathetic neurons and their associated glia. Using an inducible Cre recombinase-based fate mapping system, we have permanently marked a subpopulation of NCCs that expresses *Neurogenin-2* (*Ngn2)*, a bHLH transcription factor required for sensory neurogenesis and compared its fate to the bulk NCC population marked by expression of *Wnt1*. $Ngn2^{+}$ progenitors were four times more likely than $Wnt1^{+}$ NCCs to contribute to sensory rather than sympathetic ganglia. Within DRG, however, both *Ngn2*- and *Wnt1*-expressing cells were equally likely to generate neurons or glia. These data suggest that *Ngn2* marks an NCC subpopulation with a predictable fate bias early in migration. Taken together with previous work, these data suggest that NCCs become restricted to sensory or autonomic sublineages before becoming committed to neuronal or glial fates.

273. Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid subnuclei

Mariela Zirlinger, David J. Anderson

The mammalian brain is subdivided into cytoarchitectonically and physiologically distinct regions, this anatomical parcellation reflects a modular functional organization. An important first step in applying the tools of molecular biology to study brain function is to identify molecular markers for these subregions. In particular, no region-specific gene had been reported in the amygdala, which mediates fear responses and sexual and aggressive behaviors.

The amygdala is composed of over a dozen subnuclei. To find amygdala-specific transcripts in the mouse relative to other brain regions, we set out to search for genes differentially expressed in this region using microarray technology.

We have therefore employed microarray technology coupled with *in situ* hybridization to identify and characterize amygdala-enriched gene products compared to genes expressed in four other brain regions, including the cerebellum, hippocampus, olfactory bulb, and periaqueductal gray. In addition, we have later polished the molecular characterization of the amygdala by identifying genes differentially enriched within its various subnuclei. This was accomplished by comparing gene expression profiles from finely dissected pieces of tissue obtained by laser-capture microdissection (LCM), followed by amplification of RNA contents.

The coupling of LCM-tissue extraction, RNA amplification, microarray analysis and *in situ* hybridization inspection of identified enriched genes proved a powerful combination of techniques to characterize brain regions at an unusually high level of detail. Remarkably, we observed that the majority of amygdala-enriched genes had expression domains, whose limits coincided, at least in part, with subregion boundaries demarcated by classical histological techniques such as Nissl staining. This was the case regardless of the homogeneity of the starting material, since we obtained subregion-confined genes when the entire amygdala tissue was extracted in a rather crude manner by hand dissection, as well as when specific amygdala subnuclei were carefully dissected by laser capture. By extension, this suggests that classically defined brain nuclei may be also delineated by domains of gene expression.

274. Stem cell transplantation in a mouse model of hereditary peripheral neuropathy

Christian Hochstim

Charcot Marie Tooth Disease type I (CMT1) is the most common inherited disorder of the peripheral nervous system, affecting one in 2500 individuals. The disease is caused by mutation or overexpression of the peripheral myelin 22 (pmp22) gene in Schwann cells, which impairs their ability to myelinate axons in peripheral nerves, causing motor and sensory deficits, especially in the distal limbs. The Trembler J mouse has the same leucine to proline point mutation in the Pmp22 gene that has also been identified in patients with CMT1, and these mice have similar impairments in motor function. Histological sections of Trembler J peripheral nerve and nerve biopsies from CMT1 patients both show an increased number of Schwann cells and the failure of these cells to adequately myelinate axons. Thus, the Trembler J mouse is a good model to study potential therapeutic approaches to human CMT1.

We would like to assess the ability of stem cell populations of the peripheral (neural crest stem cells) and central (CNS stem cells) nervous systems to generate functional myelinating glia when transplanted into the sciatic nerves of Trembler J mice. Previous studies in our lab have shown that neural crest stem cells (NCSCs), isolated as a migratory population from E10.5 rat neural tubes, have the potential to generate Schwann cells and that the culturing NCSCs with the growth factor GGF promotes this fate. CNS stem cells grown as neurospheres have been shown to form peripheral "Schwann cell type" myelinating glia when transplanted into demyelinated regions of spinal cord. If these studies can show that transplantation of stem cells with gliogenic potential (CNS or PNS derived) into Trembler J mouse sciatic nerve can repair demyelinated axons and improve nerve conduction, it would support the potential use of stem cell-based therapies for human CMT1 as well as validate this approach for other genetic diseases affecting a specific cell type.

275. The involvement of the anterior cingulate cortex in trace versus delay fear conditioning demonstrated by lesion and *c*-fos mRNA *in situ* hybridization

C.J. Han,[1], L. van Trigt,[1], D.J. Anderson, C. Koch,[1]

Attention to the CS-US contingency was previously reported to be necessary for successful trace conditioning, but not for delay conditioning, and the anterior cingulate cortex has been implicated in mediating attention. We examined the involvement of the anterior cingulate cortex in mice using trace versus delay fear conditioning. Trace conditioning is different from delay conditioning in that there is a temporal gap between the end of the conditioned stimulus (CS) and the start of the unconditioned stimulus (US). It has been shown in humans that trace, but not delay, conditioning requires awareness of the CS-US contingency (1). C57BL/6N male mice were housed in the training chambers overnight and fear conditioned on the second day (6 x 16-sec 2k-Hz sine-wave tone followed by a 2-sec 0.5-mA footshock). The temporal gap between the CS and the US in the trace group was 18 sec, while the CS immediately preceded the US in the delay group. The mice were randomly divided into two groups. The first group (one out of four in each condition) was sacrificed after training for quantification of c-fos mRNA (an indicator of neuronal activity). The second group (the remaining three in each condition) was placed into a different context the following day to test the efficacy of the conditioning. The conditioning was found effective in both the delay and trace groups, compared with the US-only group ($p<0.05$). *In situ* hybridization was carried out using a cRNA digoxygenin-labeled probe and design-based stereology was used to quantify the number of c-fos positive cells. Neuronal activity in the brain during delay and trace fear conditioning were compared. The results showed that mice trained in the trace paradigm had significantly more c-fos positive cells in the anterior cingulate cortex than those trained in the delay paradigm. Furthermore, excitotoxic lesion of the anterior cingulate cortex impaired conditioned tone freezing behavior in mice that received trace conditioning, but not those that received delay conditioning.

[1]*Computation and Neural Systems, Caltech*

Reference

(1) Clark, R.E. and Squire, L.R. (1998) *Science* **280**:77-81.

276. Molecular differences between arterial and venous endothelial cells

Donghun Shin, David J. Anderson

The transmembrane ligand ephrinB2 and its receptor tyrosine kinase EphB4 are molecular markers of embryonic arterial and venous endothelial cells (ECs), respectively, and are essential for angiogenesis. In addition, several other genes are differentially expressed between arterial and venous ECs. We assumed that more genes would be differentially expressed between arterial and venous Ecs, and that identification of these genes would help us to more deeply understand the characteristics of arterial and venous ECs. Thus we applied subtractive hybridization screening to the cloning of arterial and venous-specific genes.

We dissected umbilical vessels from E11 mouse embryos, separated umbilical arteries and umbilical veins, and sorted Flk-1 and PECAM-1 double-positive ECs from the dissected arterial and venous vessels using fluorescence-activated cell sorting (FACS). We applied single-cell-based PCR amplification methods with biotinylation subtraction and differential hybridization screening. Through the 1st and 2nd rounds of subtraction and differential hybridization screening, we isolated several known and unknown genes specifically expressed in arterial Ecs. The arterial specific expressions of these genes were verified by *in situ* hybridization. The known genes include connexin37, connexin40, islet1 and IGFBP-5 protease.

One of the unknown genes is specifically expressed in arterial endothelial cells at early developmental stages such as E9.0, and the expression is down regulated at later stages of development. We found the full coding region of the gene using the Celera database. The protein does not have any motif to help identify its function. Therefore, to understand the functions of the gene, we are generating a knockout mouse.

277. Topographical and functional connections between amygdaloid and hypothalamic nuclei

Gloria Choi, David Anderson

We are interested in innate behaviors, and more specifically we are trying to understand how the circuitries in the brain controlling these innate behaviors form during development.

Two brain regions that have been implicated in controlling innate behaviors are the amygdala and hypothalamus. Classical lesion studies and studies of c-fos expression patterns after specific stimuli demonstrated that various nuclei in these two brain regions are involved in either reproductive or defensive behaviors. More interestingly, there is a topographic organization of the connections between the amygdaloid and hypothalamic nuclei in such a way that amygdaloid nuclei involved in reproductive or defensive behaviors preferentially make direct connections to hypothalamic nuclei that are also involved in reproductive or defensive behaviors, respectively.

The direct connection between two nuclei involved in the same behavioral circuitry is reminiscent of the monosynaptic connections formed between different motor neuron pools and subsets of proprioceptive sensory neurons that share common muscle targets. In this system, specific transcriptions factors are expressed both in the presynaptic sensory and postsynaptic motor neurons in a matching fashion, and these transcriptional factors, in turn, control the expressions of cadherin molecules. One can hypothesize that the matching in the expressions of cell

surface adhesion molecules, cadherins, on the axons of proprioceptive neurons and on the dendrites of motor neurons could provide the basis for the selectivity with which the connections between functionally related groups of neurons are formed in this circuit.

Analogous to the matching transcription factor expression in the motor and sensory neurons that innervate the same muscles, we want to find the genes that are expressed in the both hypothalamic and amygdaloid nuclei involved in the same behavioral circuitry. In order to do so, we are screening numerous candidate genes by *in situ* hybridization and trying to map out the projection targets of the cells that express these genes. After careful analysis, we would like to choose a best candidate gene and knock it out to study its functional role in making the specific connections between amygdaloid and hypothalamic nuclei.

278. The hmg-box factor sox10 is a determinant of stem cell properties in neural crest cells

Jaesang Kim[1], Liching Lo, Emma Dormand[2], David J. Anderson

The mechanisms that maintain stem cell lineage potentials are poorly understood. We show that the neurogenic signal BMP2 rapidly extinguishes glial potential in neural crest stem cells (NCSCs), and that constitutive expression of the HMG-box factor SOX10 prevents such extinction. Surprisingly, it also prevents extinction of neuronal potential by the myofibrogenic signal TGFβ, in part by maintaining inducibility of the proneural gene MASH1. At the same time, SOX10 inhibits overt neuronal and smooth muscle differentiation, as well as proliferative arrest induced by TGFβ. The fact that SOX10 maintains non-glial as well as glial lineage potentials in NCSCs, while inhibiting non-glial differentiation and maintaining proliferation, suggests a role in stem cell maintenance in addition to its established role in peripheral gliogenesis.

[1]*Ewha Woman's University, Seoul, Korea*
[2]*John Radcliff Hospital, Oxford, England*

279. Identification of candidate genes for neurogenic capacity and early markers for Schwann cell progenitors

Kenji Orimoto

Environmental signals (extrinsic factors) and molecular codes (intrinsic factors) are critical determinants of cell fates in stem cells. Recent arguments about the plasticity of lineage restricted stem cells have raised fundamental questions in stem cell biology: "What is a stem cell" and "How can one cell type be distinguished from another?" Thus, the stem cell is coming to an identify crisis. To address these questions, it is necessary to identify markers for stem cells at the molecular level.

Sciatic neural crest stem cells (sNCSCs) can differentiate into neurons, Schwann cells and myofibroblast in response to BMP2, GGF2/Delta, and TGF-bs. sNCSC culture is an ideal experimental system for studying stem cell biology because sNCSCs are prospectively isolated from fresh tissue without intervening culture manipulation and making it possible to analyze the cell fate at clonal level.

To get a better understanding of the molecular identity of neural crest stem cells, a cDNA subtraction was performed between E14.5 $p75^{+}P0^{-}$ sciatic nerve cells (neural crest stem cells: NCSCs) and E18.5 $p75^{+}P0^{+}$ sciatic nerve cells (Schwannomyofibroblastic progenitors: SMPs). Based on the results of sequence analyses, genes were functionally assorted into the following categories: 1) cell fate determination; 2) chromatin remodeling; 3) cell cycle machinery; 4) signal transduction; and 5) growth factors/extracellular matrix proteins.

To examine the spatio-temporary regulation of expression, *in situ* hybridization will be employed. To investigate their functional role in cell fate regulation, misexpression experiments, including loss-of-function and gain-of-function assays, will also be conducted.

280. Transynaptic tract tracing and genetic ablation of pain circuitry

Mark J. Zylka, David J. Anderson

We are interested in studying behavior at the level of molecularly defined neural circuits. We have decided to focus on pain-sensing (nociceptive) circuitry for several reasons: 1) pain sensing neurons and circuitry are found in a restricted part of the nervous system—the dorsal root ganglia; 2) there are a number of well-documented stimuli that induce pain "behaviors" in model animals (examples of stimuli include noxious heat, chemicals or wounding); 3) traditional tract tracing and neuroanatomical studies have provided a general picture of how the pain circuitry is wired peripherally and centrally, and most importantly; and 4) we have identified a large family of *Mrg* G protein-coupled receptors (Mas-related gene GPCR's) that are expressed only in restricted subsets of pain-sensing neurons.

These GPCR's represent unique molecular markers for subsets of nociceptive neurons. We will use these *Mrg* genomic loci to target tract tracing molecules (including farnesylated EGFP, alkaline phosphatase, and wheat germ agglutinin) and toxins to these nociceptive circuits. These various lines of mice will allow us to: 1) trace circuitry in the periphery and in the spinal cord; 2) transynaptically identify pain circuitry in the central nervous system; and 3) study pain behaviors in the presence or absence of the neurons (via conditionally killing or silencing the cells). Ultimately, these studies will provide us with new knowledge about the types of painful stimuli that *Mrg*-containing neurons sense as well as how *Mrg*-expressing circuits are wired at the systems level.

281. Search for genes specific for brain areas involved in innate fear responses

Walter Lerchner, Jo Del Rio, Carrolee Barlow*, David J. Anderson*

Mice respond to an aversive ultrasound differently depending on their environment and previous experience. States of higher anxiety correlate positively with a freezing response to the stimulus, while states of lower anxiety correlate with a higher number of flight responses. A study by Raymond Mongeau in the lab has mapped brain regions activated differentially in mice showing primarily freezing response vs. mice showing primarily flight responses. This study is important because it suggests antagonistic circuits in active vs. passive fear responses and might lead the way in better understanding the complexity of human anxiety disorders.

I will try to take this effort a step further by applying molecular biology to better elucidate these circuits. In the past year I have optimized a method that allows reproducible linear amplification from small amounts of RNA isolated from laser capture dissected material of very defined brain regions, such as the ventro-lateral septum or the shell of the accumbens. The amplified RNA is then used to screen Affymetrix oligo-nucleotide arrays for genes specifically expressed in regions activated by either active or passive fear responses. The expression pattern of candidate genes is confirmed by *in situ* hybridization.

Initial studies comparing only the ventro-lateral septum with the dorso-lateral septum resulted in several genes with locally restricted expression, however, we anticipate the wider study will lead to genes even more specific for important sub-regions of the differentially activated circuits.

The expression domains of these genes can then be used to either silence the neurons in these areas and/or to manipulate their neuro-transmitter release or uptake as well as trace their connections.

**Salk Institute*

282. Tripotentiality of neural stem cells?

Limor Gabay

Among the many contradictory reports about the plasticity of neural stem cells, one result that is agreed upon is that all CNS stem cells can differentiate into at least three cell types: neurons, astrocytes and oligodendrocytes. However, this doesn't seem to be the case when one examines the mouse spinal cord *in vivo*.

During embryonic development the mouse spinal cord cells are differentiated in the ventral-dorsal axis into distinct domains marked by gene expression which give rise to different subtypes of neurons and glia. It was found that each group of progenitors generate *in vivo*, either neurons and astrocytes or neurons and oligodendrocytes, but not all three cell types, in contrast to the tripotentiality of these cells when grown *in vitro* (Zhou and Anderson, 2002).

I am trying to understand the reason of this inconsistency. To this end I'm isolating two groups of progenitors from the spinal cord: one from the dorsal part and one from the ventral. In the ventral part the progenitors are marked by the expression of the Olig2 gene and can give rise to motoneurons and oligodendrocytes. Expression of the GFP gene under the Olig2 promoter control is used to select for these cells (Zhou and Anderson 2002). The cells from the dorsal part do not express Olig2 and give rise *in vivo* to different subtypes of neurons and astrocytes, but not to oligodendrocytes and motoneurons.

After growing the cells *in vitro* in a neurosphere culture, I found that the expression of GFP is maintained in most of the cells grown from the O2-positive cells, but surprisingly, it also appears in some of the cells from dorsal region. Induction of Olig2 expression in some the dorsal cells probably result in their ability to generate oligodendrocytes, while loss of Olig2 in some of the ventral cells may allow differentiation into astrocytes. I am checking whether these events may explain the tripotentiality of these CNS progenitor cells *in vitro*.

283. Functions of mammalian *achaete-scute* and *atonal* homologous in generic neuronal differentiation and subtype specification

Liching Lo, David J. Anderson

In *Drosophila*, proneural genes, such as *achaete-scute* and *atonal*, can promote generic neuronal differentiation, and they can also specify neuronal subtype identity. In the fly PNS, *ac-sc* specifies external sensory (ES) organ identity, while *ato* primarily specifies chordotonal (CD) organ identity. However, *ato* can specify ES as well as CD identities, depending on cell context, while *ac-sc* exclusively specifies ES identity. The vertebrate homologous of proneural genes, such as *ato*-related genes, *neurogenins*, and the *ac-sc* related *Mash1*, are required for neurogenesis in distinct lineages and are sufficient to promote generic neuronal differentiation. The question remains whether these vertebrate homologous can specify neuronal subtype identity as well. Although evidence from *in vivo* have shown *Mash1* or *neurogenins* can promote subtype identities, it is not clear whether the genes autonomously promote such functions or the environment is simply permissive for such phenotypic expression. There is also no data on a direct comparison of *Mash1* and *neurogenins* in subtype specification in an environment that can be manipulated. I have misexpressed *Mash1* and *neurogenins* in early neural crest cells derived from either the neural tube or in neural crest stem cells (NCSCs). In neural tube progenitors dorsalized by low concentrations of BMP2, NGNs promote exclusively sensory neurogenesis, while MASH1 promote only autonomic neurogenesis. However, at higher concentrations of BMP2, or in NCSCs, NGNs promote only autonomic neurogenesis. These data suggest that the identity specification functions of NGNs is more sensitive to context than is that of MASH1. In NCSCs, MASH1 is more sensitive to Notch-mediated inhibition of neurogenesis and cell cycle arrest. Taken together, our data may explain cellular differences between MASH- and

NGN-dependent lineage in the timing of neuronal differentiation and cell cycle exit.

284. Changes in brain activity following a switch in defense behaviors to a fear stimulus

R. Mongeau, E. Chiang, D.J. Anderson

A new animal model is proposed to study active vs. passive innate fear reactions. A group of mice were sensitized with foot-shocks, while another group (naive) was not. The next day mice were exposed to an ultrasound stimulus that was found to elicit unconditioned fear reactions. Sensitized mice displayed enhanced freezing and decreased flight reactions to this unconditioned stimulus compared to naive mice. Furthermore, naïve mice placed in a new cage displayed a similar switch in defense reactions to the aversive acoustic stimulus. A negative correlation was found between flight and freezing suggesting that these behaviors are competing motor expression of fear. To map the neural substrates underlying this behavioral switch, c-fos mRNA (an indicator of neural activity) using *in situ* hybridization with single-cell resolution was carried out, allowing design-based stereology quantification to be successfully used to assess changes in the brain. Mice displaying more freezing than flight to the aversive acoustic stimulus had more neuronal activity in the lateral septum and in various hypothalamic nuclei in the vicinity of the third ventricle. However, these sensitized animals had reduced neuronal activity, compared to naïve controls, in delimited areas of the limbic system and the basal ganglia. The above results are discussed in view of previously established animal models of fear and a functional circuit regulating active and passive defense is proposed.

285. Olig genes and the oligodendrocyte-astrocyte fate decision

Sally Lowell

There are two major types of glial cells in the central nervous system: astrocytes and oligodendrocytes. Oligodendrocytes emerge from a well-defined ventral region of the spinal cord that is precisely demarkeated by the expression of the bHLH transcription factors Olig1 and Olig2. Astrocytes emerge from a much broader domain that is poorly defined but may encompass most regions of the spinal cord with one exception: astrocytes appear not to arise from the domain that expresses Olig1 and Olig2 (Zhou and Anderson, 2002). Astrocytes and oligodendrocytes, therefore, appear to arise from separate progenitor populations *in vivo*. Those progenitors, however, when removed from the spinal cord and placed in culture, have the potential to generate both types of glia from a single cell. This raises the question of how these progenitors are restricted to generating only oligodendrocyte or only astrocytes when *in vivo*.

Recent data points to a central role for the Olig genes in regulating this astrocyte-oligodendrocyte fate decision. Firstly, gain of function studies in chick embryos demonstrated that Olig2 is sufficient to cause ectopic differentiation of oligodendrocytes when missexpressed together with the HD factor Nkx2.2 (Zhou *et al*., 2001). Secondly, targeted disruption of Olig1 and Olig2 in mice results in a complete elimination of oligodendrocytes, with the progenitors that would normally generate oligodendrocytes instead generating astrocytes (Zhou and Anderson, 2002). This data suggests two roles for Olig genes during gliogenesis: promotion of oligodendrocyte fate and suppression of astrocyte fate, and raise the question of whether these two roles are independent from each other or not. I am using a gain of function approach in primary CNS stem cells in order to investigate the mechanisms by which Olig1 and Olig2 promote oligodendrocyte differentiation and suppress astrocyte differentiation, and to ask how olig genes interact with other signaling pathways that are thought to regulate astrocyte differentiation.

286. A diverse family of GPCRs expressed in specific subsets of nociceptive sensory neurons

Xinzhong Dong, David J. Anderson

To study pain at the molecular level, we searched for genes specifically expressed in nociceptors. We identified a gene family called *mrgs*, which has nearly 50 members. Many of them are only expressed in a subset of nociceptors defined by lectin IB4 labeling, but not in any other tissues in the body. The IB4+ nociceptors have been implicated in nerve injury induced pain, also called neuropathic pain. Interestingly, the expression of different *mrg* genes is largely non-overlapping, suggests there is unexpected molecular diversity among the IB4+ nociceptors. The mrg family belongs to G protein-coupled receptors superfamily. We found the mrgs function as neuropeptide receptors *in vitro*.

Although our results suggest the *mrg* family plays a role in pain sensation, there are many unanswered questions about their functions. We are currently generating knockout mice in which the entire *mrgA1* or *mrgD* open reading frames will be deleted and replaced by an axonal tracer. We will conduct various pain behavioral assays on these mutant mice, to determine whether these genes have direct or regulatory roles in pain sensation. Second, with the axonal tracer under *mrg* endogenous promoters in these mice, we will be able to determine whether the different *mrg* expressing nociceptors project to different target regions in periphery and spinal cord, and whether these genes are involved in axonal guidance. We are also making transgenic mice in which *mrgA1* will be ectopically expressed in a normally non-*mrg* expressing subpopulation of nociceptors. We will determine whether the ectopic expression of *mrgA1* in these nociceptors will change their cellular properties and have any effects in pain sensitivity. In addition, we are searching the enhancer elements of *mrg* genes, which are responsible for the nociceptor-specific expression. The isolation of tissue-specific enhancer elements is very important for studying gene regulation. The enhancer elements are also great molecular tools for other functional studies.

287. Peripheral nerves mediate arterial differentiation and pattern of vascular branching

Yosuke Mukoyama

Nerves and blood vessels are known to be branched structures. An interesting question is whether the patterning of nerve and blood vessel growth is established independently or interdependently. Both neurons and blood vessels share similar molecules that include ephrins/Eph, Neuropilins (Semaphorin receptor) and Notch molecules, prompting further study of their relationship.

Using a series of arterial-specific markers, we have discovered that arteries, but not veins, are specifically aligned with peripheral nerves in embryonic limb skin. Analysis of *ngn1/ngn2* double mutants or *erbB3* mutants that eliminate peripheral nerves or Schwann cells, respectively, shows that arterial differentiation as well as vascular branching is dependent on the presence of such nerves. Although *sema3A* mutants show disorganized nerves, blood vessels are shown to associate with the disorganized nerves and develop arterial marker induction as normal. Taken together, the pattern of arterial branching is determined by that of the nerve. The nerve-derived signal may be VEGF that is necessary and sufficient to mediate arterial differentiation *in vitro*. We are currently investigating VEGF involvement *in vivo* by examining conditional VEGF knockout mice that completely lack VEGF expression in peripheral nerves. We are the first to demonstrate a requirement for a peripheral nerve-derived signal, VEGF, in the patterning of vessels and differentiation of arteries from veins. Furthermore, we suggest a new concept in angiogenesis, that is that local signals provided by tissue sub-component, such as peripheral nerves, may provide a template that establish tissue-specific pattern of vascularization.

288. Searching for downstream targets of Olig1,2 genes

Qiao Zhou, Christian Hochstim, David Anderson

Oligodendrocytes are the myelinating glial cells of the vertebrate central nervous system (CNS). During the development of the embryonic spinal cord, oligodendrocyte precursors arise from a specific ventral focus of the ventricular zone that also generates motoneurons at an earlier time. Two members of a recently described bHLH transcription factor family, Olig1 and Olig2, are sequentially expressed first in motoneuron precursors, and then in oligodendrocytes. To investigate the role of Olig genes in the development of these two cell types, we used targeted deletion to simultaneously inactivate both Olig1 and Olig2 in the mouse. Interestingly, in the Olig1,2$^{-/-}$ double null spinal cord, motoneurons are fate transformed to V2 interneurons, while oligodendrocyte is respecified to an astrocyte fate.

The observation that Olig genes suppress astrocyte development is intriguing. Together with our previous demonstration that Olig2 acts as a transcriptional repressor in the ectopic induction of oligodendrocytes in the chick embryos, these data suggest that Olig genes promote oligodendrocyte fate indirectly via suppression of alternative cell fates. We are therefore interested in searching for downstream target genes regulated by Olig1,2. Taking advantage of the fact that a Histone-GFP fusion is knocked into the Olig2 locus, we sorted out GFP^{+} cells from both heterozygous and homozygous E14.5 mouse spinal cord by FACS. The mRNA expression profiles of the Olig1,2$^{+/-}$ and Olig1,2$^{-/-}$ cells will be compared with AFFYMETRIX mouse gene chips. We believe that this analysis will help us to gain further insight into the gene regulatory networks that regulate the generation of the glial cells.

289. Genetic and molecular mechanism of avoidance behavior ("fear") in *Drosophila*

Greg S. Suh, Anne Simon, Seymour Benzer, David J. Anderson

I have developed a novel behavioral paradigm that monitors innate avoidance (fear) response in *Drosophila* in collaboration with the Seymour Benzer laboratory. In this paradigm, fruit flies (testers or responders) avoid the tube in which a set of flies (shakers or emitters) were previously traumatized by shaking or vortexing. It is important to note that flies do not necessarily avoid a tube that was occupied by a set of flies for several hours. As fruit flies are known to secrete chemical stimuli to induce behavioral changes of others (e.g., Pheromone), I hypothesize the following: *flies produce and emit "alarm" signals in response to traumatic stimuli* and that *production/emission of the signal requires a specific neural circuit*, and that *responder flies activate a specific neural circuit to mediate avoidance response upon recognition of "alarm" signal.*

Responder flies were given a choice between a fresh tube and a shaken tube with flies in T-maze. Most responders chose the fresh tube; the Performance Index (PI) (a measure of avoidance of the tube with shaker flies) lies between 95 and 85 in optimal conditions. As the avoidance response is robust, we will be able to initiate a genetic screen to isolate a set of mutants failing to emit, and another set failing to avoid, the "alarm" signal. We are currently also mapping the neural circuit governing the avoidance behavior. I have received a collection of Gal4 enhancer trap lines expressed in specific regions of the adult brain from the Kim Kaiser laboratory. I will cross them to UAS-*shibire*^{ts} to assay their avoidance behavior at the non-permissive temperature, and compare to their performance at the permissive temperature. I will use them as both emitters and responders. Thus, I would isolate a set of Gal4 lines defective in emitting "alarm" signal and another set failing to avoid the shaken tube. Once I isolate them, I will cross the Gal4 lines to UAS-farnesyl-GFP, a membrane anchored form of GFP, or UAS-cytoplasm *lacZ* to visualize neural circuits governing emission of the signal and avoidance response.

Publications

Anderson, D.J. (2001) Stem cells and pattern formation in the nervous system: The possible vs. the actual. *Neuron* **30**:19-35.

Anderson, D.J., Gage, F.H. and Weissman, I.L. (2001) Can stem cells cross lineage boundaries? *Nature Med.* **7**:393-395.

Chen Z.F., Rebelo, S., White, F., Malmberg, A.B., Baba, H., Lima, D., Woolf, C.J., Basbaum, A.I. and Anderson, D.J. (2001) The paired homeodomain protein DRG11 is required for the projection of cutaneous sensory afferent fibers to the dorsal spinal cord. *Neuron* **31**:59-73.

Dong, X.Z., Han, S., Zylka, M.J., Simon, M.I. and Anderson, D.J. (2001) A diverse family of GPCRs expressed on specific subsets of nociceptive sensory neurons. *Cell* **106**:619-632.

Gerety, S.G. and Anderson, D.J. (2001) Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* **129**:1397-1410.

Kubu, C.J., Orimoto, K., Morrison, S.J., Weinmaster, G., Anderson, D.J. and Verdi, J.M. (2002) Developmental changes in Notch1 and Numb expression mediated by local cell-cell interactions underlie progressively increasing delta sensitivity in neural crest stem cells. *Dev. Biol.* **244**:199-214.

Lo, L.C., Dormand, E., Greenwood, A. and Anderson, D.J. (2002) Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian *achaete-scute* and *atonal* homologs in cultured neural progenitor cells. *Development* **129**:1553-1567.

Mukouyama, Y., Shin, D., Britsch, S.T., Masahiko, T. and Anderson, D.J. (2002) Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109**:693-705.

Shin, D., Garcia-Cardena, G., Hayashi, S.I., Gerety, S., Asahara, T., Stavrakis, G., Isner, J., Folkman, J., Gimbrone, Jr., M.A. and Anderson, D.J. (2001) Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev. Biol.* **230**:139-150.

Weissman, I.L., Anderson, D.J., and Gage, F.H. (2001) Stem and progenitor cells: Origins, phenotypes, lineage commitments and transdifferentiations. *Annu. Rev. Cell Dev. Biol.* **17:**387-403.

White, P.M., Morrison, S.J., Orimoto, K., Kubu, C.J., Verdi, J.M. and Anderson, D.J. (2001) Neural crest stem cells undergo cell-intrinsic developmental changes in sensitivity to instructive differentiation signals. *Neuron* **29**:57-71.

Zhou, Q. and Anderson, D.J. (2002) The bHLH transcription factors Olig2 and Olig1 couple neuronal and glial subtype specification. *Cell* **109**:61-73.

Zhou, Q., Cho, G. and Anderson, D.J. (2001) The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. *Neuron* **31**:791-807.

Zirlinger, M., Kreiman, G. and Anderson, D.J. (2001) Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid sub-nuclei. *PNAS* **98**:5270-5275.

Zirlinger, M., Lo, L.C., Mcmahon, J., Mcmahon, A.P. and Anderson, D.J. (2002) Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. *PNAS* **99**:8084-8089.

James G. Boswell Professor of Neuroscience, Emeritus (Active): Seymour Benzer
Visiting Associate: Carol A. Miller, Atsushi Yamaguchi
Postdoctoral Fellows: Ted Brummel, Pankaj Kapahi, Debbie Liang, Laurent Seroude, Anne Simon, W. Dan Tracey Jr., David W. Walker, Horng-Dar Wang
Research and Laboratory Staff: Stephanie Cornelison, Anh Dinh, Amparo Gomez, Nick Lawrence, Viveca Sapin, Ching-Hua Wei, Rosalind Young, Ute Zimmerman
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Summary: Our group uses *Drosophila* as a model system in which to identify and isolate genes involved in behavior, aging, and neurodegeneration and to analyze their basic functions. The high degree of homology between the fly and human genomes forms the basis of this strategy for understanding the corresponding human genes.

A major interest is the problem of aging, and the single-gene approach is a direct and rapid method of screening for mutants with enhanced longevity. For instance, the mutant, *methuselah*, which extends the average lifespan of *Drosophila* by some 30%, also provides increased resistance to different stresses, including heat, starvation, and the oxidative damage induced by paraquat. We have undertaken a screening program for mutants that are resistant to stress, as genes that are induced or repressed by different forms of stress may identify regulatory pathways relevant to aging. Exposure of flies to 100% oxygen causes early death, and we have found that an early event induced is local disruption of mitochondrial structure in the form of internal "swirls." These also accumulate in normal aging, and their formation can be suppressed by certain genes. Caloric restriction is a well-known method of extending lifespan in various organisms, and we are investigating that in *Drosophila*, as well as the role of steroid hormones, and the role of bacterial flora. We are developing biomarkers to monitor the progress of aging during lifetime. And we have shown that fly lifespan can be extended by simple feeding of a drug which alters the balance of induction and repression of different sets of genes.

Flies, like humans, suffer from a variety of hereditary neurological diseases. One approach has been to isolate mutants with shortened lifespan, thus identifying genes whose functions are needed to maintain the integrity of the nervous system against late-onset deterioration. This has led to a series of mutants such as *drop-dead, spongecake, eggroll* and *bubblegum*, the names describing structural changes that appear with aging in the mutant brains, as seen by electron microscopy. The hereditary defects in some of these mutant brains resemble those seen in various age-related diseases in humans. For instance, *spongecake* has inflated axon terminals, as in Creutzfeldt-Jakob disease. In *bubblegum*, there is visual system degeneration associated with an excess of very long-chain fatty acids, as in human adrenoleukodystrophy, and the degeneration can be prevented by feeding the mutant with one of the ingredients of "Lorenzo's oil." As in humans, lipofuscin-like fluorescent material accumulates in the fly nervous system with age, and we use genetics to study the formation of such waste material and its disposal.

At least eight hereditary neurological diseases in humans are associated with the presence in the causative genes of expanded stretches of triplet nucleotide repeats. The consequently coded polypeptides create nuclear inclusions that apparently underlie neuronal degeneration. We have reconstituted this phenomenon in the compound eye of the fly and searched for genes whose expression can counteract the toxic effects of polyglutamine repeats. Several such suppressor genes have been cloned, and their action verified in transgenic flies.

Our group is also investigating nociception in *Drosophila*, which bears much resemblance to human pain. Mutants such as *painless* represent an entry into a molecular genetic analysis of this phenomenon.

290. Steroid control of longevity in *Drosophila melanogaster*

Anne F. Simon, Cindy H. Shih, Seymour Benzer

We have found that different components of the steroid pathway, from the ligand to the receptor, can be manipulated to extend longevity. Flies heterozygous for mutations of a gene involved in the biosynthetic pathway of ecdysone, the major steroid in flies, or mutations affecting the ecdysone receptor (*EcR*), increase longevity by around 50%. The longevity extension can be suppressed by feeding ecdysone.

Under our conditions of mild down-regulation in heterozygous mutant flies, we observed no reduction in fertility or fecundity. Thus, it is possible to adjust expression levels of genes involved in reproduction to increase longevity, without necessarily causing a deficiency in reproductive output.

It is known that *EcR* and *ecdysonless* (another gene of the biosynthetic pathway) are involved in adult nervous system development (Schubiger *et al*., 1998; Lee *et al*., 2002), and *EcR* is expressed in at least in some neurons of the young adult (Robinow *et al*., 1993). We confirmed expression of *EcR* throughout the central nervous system in 3-4 day old adults. EcR heterozygous mutants show improved behavioral performance in an activity test, so modulating the steroid level could have a protective effect on neurons, as observed for the insulin pathway in *C. elegans* (Wolkow *et al*., 2000).

We conjecture that the overall effect of mutations in our study is to change the balance between repression and activation of transcription of sets of target genes. The effects can be long term, as indicated by our observations, using temperature-sensitive mutations, that being mutant for only part of early adult life is enough to induce an increase in longevity.

But which genes are crucial for the observed effects? During development, *EcR* regulates the expression of numerous genes, some of which known to be involved in longevity regulation (Finch and Ruvkun, 2001), e.g., the chaperones hsp27 and 23 (Luo *et al*., 1991) and catalase (Radyuk *et al*., 2000).

We plan to perform a chip analysis study on the biosynthesis mutant, fed or not with ecdysone, to determine the pattern of gene expression modification. We will also investigate, at a physiological level, what is different in the mutant flies that allows them to live longer. It will also be of interest to study interactions with other known longevity mutants, as *methuselah* (Lin *et al*., 1998), Indy (Rogina *et al*., 2000), and mutations affecting the insulin pathway.

References

Finch, C.E. and Ruvkun, G. (2001) *Ann. Rev. Genomics Human Genet*. **2**:435-462.

Hodin, J. and Riddiford, L.M. (1998) *Dev. Genes Evol*. **208**:304-317.

Lee, T., Marticke, S., Sung, C., Robinow, S. and Luo, L.Q. (2000) *Neuron* **28**:807-818.

Lin, Y.J., Seroude, L. and Benzer, S. (1998) *Science* **282**:943-946.

Luo, Y., Amin, J. and Voellmy, R. (1991) *Mol. Cell. Biol*. **11**:3660-3675.

Radyuk, S.N., Klichko, V.I. and Orr, W.C. (2000) *Arch. Insect Biochem. Physiol*. **45**:9-93.

Robinow, S., Talbot, W S., Hogness, D.S. and Truman, J.W. (1993) *Development* **119**:1251-1259.

Rogina, B., Reenan, R.A., Nilsen, S.P. and Helfand, S. L. (2000) *Science* **290**:2053-2062.

Schubiger, M., Wade, A.A., Carney, G.E., Truman, J.W. and Bender, M. (1998) *Development* **125**:2053-2062.

Walker, V.K., Watson, K.L., Holden, J.J.A. and Steel, C.G.H. (1987) *J. Insect Physiol*. **33**:137-142.

Wolkow, C.A., Kimura, K.D., Lee, M.-S. and Ruvkun, G. (2000) *Science* **290**:147-150.

291. Genetics and pathology of oxygen toxicity in *Drosophila*

David W. Walker, Colin Rundel, Seymour Benzer

Molecular oxygen is a biradical that, upon single electron additions sequentially generates O_2^-, H_2O_2 and OH which, by further reactions, can generate an array of additional reactive oxygen species (ROS). We have examined the underlying pathology associated with oxygen toxicity in *Drosophila*. Using electron microscopy, we have identified a striking initial pattern of degeneration of the mitochondria within flight muscle under conditions of oxidative stress; cristae within individual mitochondria become locally rearranged in a fashion that we have termed *swirls*. To examine the functional relevance of mitochondrial *swirls*, we used a staining technique which can identify respiratory enzyme cytochrome *c* oxidase (COX) activity, using electron micrographs to identify COX activity at the individual mitochondrial level. We found that cristae involved in a *swirl* are COX^- deficient within an otherwise COX^+ mitochondrion.

To investigate the relationship of mitochondrial *swirls* to aging under normoxic conditions, we assayed *swirl* formation with age in normal lifespan strains, finding that *swirls* accumulate in old individuals. Furthermore, the long-lived *methuselah* mutant displays increased survival under hyperoxia and fewer mitochondrial *swirls*. A logical extension of the free radical theory of aging focuses attention on the mitochondria. The mitochondrial electron transport chain consumes 85% of the oxygen utilized by the cell. We are currently examining mitochondrial physiology under conditions of oxidative stress, using biochemical, physiological, and molecular techniques.

A second approach involves the molecular cloning of key molecules involved in oxygen defense. We screened 800 UAS-containing (EP) insertion lines for hypersensitivity to oxygen stress, and identified 15 mutant lines that display unusually high sensitivity to hyperoxia. These are of great interest, as the disrupted genes are ones necessary for coping with oxidative stress. Some of these genes, when overexpressed using the UAS/GAL4 system, lead to hyperoxia resistance and extended lifespan. We are characterizing these genes and focussing on their role in maintaining age-related mitochondrial integrity.

292. Nutrition-modulation of lifespan in *Drosophila*: The roles of Sir2 and fatty acid amide hydrolase

Pankaj Kapahi, Brian Zid, Seymour Benzer

Enhancer-promoter (EP) transposable elements were employed for a gain-of-function screen to identify genes which, upon overexpression, lead to increased longevity in *Drosophila melanogaster*. We isolated seven strains that have lifespans extended by at least 25%, including Sir2 and a gene that has homology to human fatty acid amide hydrolase (FAAH). Next, we examined the roles of these genes under varying conditions of nutrition.

In *Drosophila*, as in the mouse, overnutrition causes a decrease in lifespan; a striking decrease in lifespan occurs as the concentration of yeast in the food is increased from 1% to higher levels. At 10% yeast, the average lifespan is halved. We examined both overexpressor and loss-of-function (LOF) strains of Sir2 and FAAH at different yeast concentrations. In 1% yeast, overexpression of Sir2 had no significant effect on lifespan, but it dramatically suppressed, the deleterious effect of overnutrition caused by feeding 10% yeast, increasing lifespan under those conditions by greater than 40%. With the Sir2 LOF strain, there was a slight decrease

in lifespan with flies on 1% yeast, but on 10% yeast there was a drastic 70% decrease in lifespan. Corresponding results were obtained for overexpressor and LOF strains of FAAH. Both these genes therefore play important roles in protecting against overnutrition. Larvae of Sir2 and FAAH LOF strains, under overnutrition, even fail to develop to adulthood. This inhibition of development can be rescued by adding trichostatin A, which inhibits RPD3, a histone deacetylase. We are performing microarray analysis in strains with different levels of Sir2 expression under different nutritional states, to identify the modulation by nutrition of downstream target genes.

293. Isolation of life extension genes by multiple stress screening, cDNA subtractive hybridization, and microarray

Horng-Dar Wang, Seymour Benzer

Long-lived organisms often are more resistant to stress. The *Drosophila* mutant, *methuselah*, which has 35% extended lifespan, resists all of three different stresses, paraquat, starvation, and heat. to find other longevity genes, we screened for ones that show increased expression in response to all three of these stresses by using cDNA subtraction and DNA microarray. Several such genes were found. One, heat shock protein 70 (*hsp70*), has been reported to extend *Drosophila* lifespan when overexpressed, supporting our hypothesis that genes that respond to multiple stresses may increase lifespan. Two other genes from our screen were *hsp26* and *hsp27*. These were used to generate transgenic lines and tested for overexpression in the UAS/GAL4 system. RT-PCR analysis confirmed that the Hsp26 and Hsp27 transgenic flies, when crossed with GAL4 driver lines, do overexpress *hsp26* and *hsp27*, and such transgenic flies have mean lifespans extended by an average of 25-32%. The transgenic flies show increased resistance to all three stresses.

We checked the effects of *hsp26* and *hsp27* overexpression on other genes known to be involved in longevity: *mth*, *Cu/Zn sod*, and *catalase*. Semi-quantitative RT-PCR detected no changes in *Cu/Zn sod* or *catalase*, but decreased expression of *mth*. This suggests the possibility that the Hsp26- and Hsp27-mediated increased lifespan in *Drosophila* may be via down-regulation of the *mth* gene.

The results demonstrate that multiple stress screening, combined with cDNA subtraction and microarrays, provide a useful approach to finding genes that may help to delineate longevity pathways.

294. A role for bacteria in regulating lifespan in *Drosophila*

Ted Brummel, Laurent Seroude, Seymour Benzer

The aging process is frequently described via lifespan curves, which fail to address the actual causes of death. Recently, work in our laboratory and elsewhere has provided evidence that one of the major changes associated with age is a late, dramatic increase in the expression of genes involved in fighting infection. This raises the possibility that one cause of death may be sepsis. Consistent with this possibility, it has been shown that the long-lived nematode strain *Age-1* accumulates bacteria at a slower rate than wild type. Thus, it is important to understand the nature of the microorganisms present in the fly.

To address this question, we have examined the levels of bacteria in flies under the usual conditions for measuring lifespan, and confirmed that a large increase in bacterial content occurs with age, relative to controls. We studied lifespan of adult flies either in the presence or absence of antibiotics. For *methuselah* and controls, there was little difference. However, for another long-lived strain, the antibiotic caused a reversal of its lifespan to the normal level, indicating that in certain genotypes, the presence of bacteria can be beneficial in extending lifespan. These provocative observations are being followed up by raising flies with and without controlled introduction of various bacterial strains.

295. Lipofuscin in *Drosophila melanogaster*

Atsushi Yamaguchi, David W. Walker, Seymour Benzer

Lipofuscin is a brown-yellow, autofluorescent, lipoidal, electron-dense material that accumulates within the lysosomal compartment of cells, including neurons. The progressive accumulation of lipofuscin is one of the most characteristic and consistent morphological cellular changes in aging. In human neuronal ceroid lipofuscinosis (NCL), a group of fatal neurodegenerative disorders characterized by blindness, seizures, ataxia, dementia and premature death, a common feature is widespread neuronal death associated with lysosomal accumulation of lipofuscin-like autofluorescent material.

To investigate the underlying mechanism of lipofuscinogenesis, we are using the fruit fly *Drosophila melanogaster* as a model system. By fluorescent microscopy, confocal laser scanning microscopy and spectrophotometry, one can see that lipofuscin accumulates in an age-dependent manner in the central nervous system (CNS) of adult flies. In certain short-lifespan mutants, it accumulates at an accelerated rate. We identified the mutant *pancake*, which displays hypersensitivity to O_2 stress, short lifespan, a defect in motor activity, and a striking accumulation of electron-dense bodies within glial cells of the peripheral brain. By studying much mutants, and isolating genes that suppress the accumulation, we will attempt to understand the mechanisms that are normally required to rid cells of such accumulated waste material.

296. Identification of a long-life gene expressed in sensory neurons

Ted Brummel, Laurent Seroude, Pankaj Kapahi, Seymour Benzer

An enhancer tap screen was performed to generate lines expressing LacZ under the control of various enhancers. 180 such lines were monitored for time-dependent changes in LacZ expression throughout their lifespan. Over 80% showed age-dependent changes in expression, the vast majority decreasing with age. We chose to focus on ones in which LacZ expression either increased with age, or declined rapidly. Since it is likely that the enhancer trap lines produce mutations that affect the genes whose expression they represent, we tested a subset of these lines for increased longevity. Of 25 insertions showing increased expression with age, none caused extension of lifespan. However, two of 40 lines with rapid drops in expression showed greater than 30% increase in lifespan. We report the characterization of one of these lines, number 817.

The P-element in the enhancer trap strain was mapped by plasmid rescue to the third exon of the olf-186F gene, which is predicted to be involved in olfaction. We generated P-element excision alleles of this line to determine whether the P insertion is responsible for the phenotype, and obtained ones that were confirmed by PCR to be precise excisions. Excision lines that continued to behave as the original P-strain were shown by PCR to be imprecise excisions. Expression of LacZ in 817 was analyzed in serial sections of the entire body, to determine which tissues were involved. Only a small subset of tissues revealed any staining, many corresponding to sensory organs. Since it has been shown that ablation of sensory neurons in *C. elegans* can lead to life extension, it is possible that similar mechanisms affect aging in both systems. The gene will be further studied to analyze its function in extending lifespan.

297. Antioxidant enzymes in aging

Debbie Liang, Seymour Benzer

According to the free radical theory of aging, aging results from an imbalance between oxidant production, antioxidant defenses, and repair processes, leading to an accumulation of oxidative damage by-products. How do these products, the response to them, or the failure to respond, ultimately determine lifespan? Development of methods to measure free radical levels will be key to understanding normal aging and altered aging in longevity mutants.

Superoxide dismutase (SOD), an antioxidant enzyme that coverts superoxide to hydrogen peroxide, exists in two forms: Cu/Zn SOD in the cytoplasm and Mn SOD in the mitochondria. In *Drosophila*, there are two common alleles of Cu/Zn SOD resulting in two different electromorphs, SOD-slow and SOD-fast. The *in vitro* activity of the SOD-slow electromorph is three times greater than that of SOD-fast, but SOD-fast is more thermostable (Lee *et al.*, 1981). In a study of genetically heterogeneous *Drosophila* populations that had been selected for late-life reproduction, the SOD-S allele was enriched in the long-lived strains (Tyler *et al.*, 1993). We are examining these alleles in long-lived single gene mutants to determine the impact, if any, of the individual SOD electromorphs on longevity and stress resistance.

References

Lee *et al.* (1981) *Proc. Natl. Acad. Sci.* USA **78**:7052-7055.

Lin *et al.* (1998) *Science* **282**:943-946.

Trapp *et al.* (1983) *Biochem. Biophys. Res. Commun.* **112**:602-605.

Tyler *et al.* (1993) *Genetica* **91**:143-149.

298. Genetic analysis of nociception

W. Dan Tracey Jr., Seymour Benzer

Using the fruit fly *Drosophila* melanogaster as a model system to understand the molecular basis of pain, we have discovered that *Drosophila* larvae respond behaviorally to noxious stimuli in a highly reproducible and stereotypical fashion. Fly larvae move through their environment with a rhythmic, peristaltic motion. When touched lightly with an innocuous probe, they briefly pause, then resume motion. But, when touched with a probe heated to an unpleasant temperature, the larvae respond by writhing vigorously sideways in a corkscrew-type motion. We are using this stereotyped behavior to perform genetic screens with the goal of identifying mutants that are insensitive or sensitized to noxious stimuli. We have focused on one such *Drosophila* mutant insensitive to noxious heat, which we have named *painless*. The mutant larvae show a delayed response to the noxious heat stimulus. With a stimulus that causes wild-type larvae to respond in less than one second, *painless* mutant larvae require an average of six seconds of stimulation to generate the response. The *painless* phenotype is caused by the insertion of a P-transposable element which has allowed us to rapidly identify the affected gene. The predicted protein is a member of the transient receptor potential family of ion channels and is distantly related to the vanilloid receptor, a molecule implicated in vertebrate pain pathways (Caterina *et al.*, 1997). To verify that we have correctly identified the *painless* gene we rescued the mutant phenotype with an 8.5 kilobase fragment of genomic DNA.

The *painless* gene is expressed in a subset of neurons in the peripheral nervous system. These cells, known as multi-dendritic neurons, have finely branched, naked processes beneath the larval epidermis, resembling the naked nerve endings of nociceptors in vertebrate skin. Blocking the function of the md-neurons with tetanus toxin causes a painless phenocopy, suggesting that the multi-dendritic neurons are the nociceptors. To demonstrate that these neurons respond to heat in the noxious range, we are using a genetically-encoded calcium sensor to visualize responses of identified neurons to the noxious heat stimulus in wild-type and mutant larvae.

Reference

Caterina, M.J., Schumacher, M.A., Tominaga, M., Rosen, T.A., Levine, J.D. and Julius, D. (1997) *Nature* **389**:816-824.

Publications

Kazemi-Esfarjani, P. and Benzer, S. (2002) Suppression of polyglutamine toxicity by a *Drosophila* homologue of myeloid leukemia factor 1. *Human Mol. Genet*. **11**:2657-2672.

Seroude, L., Brummel, T., Kapahi, P. and Benzer, S. (2002) Spatio-temporal analysis of gene expression during aging in *Drosophila melanogaster.* *Aging Cell* **1**:47-56.

Simon, A.F., Shih, C., Mack, A. and Benzer, S. (2002) Steroid control of longevity in *Drosophila melanogaster*. Submitted.

Song, W., Ranjan, R., Dawson-Scully, K., Bronk, P., Marin, L., Seroude, L., Lin, Y.-J., Nie, Z., Atwood, H. L., Benzer, S. and Zinsmaier, K.E. (2002) Presynaptic regulation of neurotransmission in *Drosophila* by the G-protein coupled receptor Methuselah. *Neuron* **36**:105-119.

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Professor of Biology: William J. Dreyer
Research Scientist: Janet Roman-Dreyer
Research Staff: Brandon King[1]
Programming Consultants: Jason Gripp, Aaron Moore
[1]*Undergraduate, Pasadena City College*

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Summary: Experimental embryologists can observe single cells as they migrate and join other cells during the sculpturing of an embryo. The specificity and precision of these events is truly extraordinary. Migrating cells coalesce to create the heart. Cells originating in the neural crest migrate and continue to divide as they contribute to the shaping of teeth – and their innervation. The genetic programs responsible are so extraordinary that the upper and lower sets of teeth fit together, and even more amazingly the teeth (and indeed all other parts) of identical twins are -- well -- nearly identical.

Half a century ago Caltech's Roger Sperry showed that the growth cones of regenerating nerves have very great specificity. His results and the other examples of the specificity of migrating cells are so remarkable that even today some scientists find it hard to believe that the cell surface molecular codes are that precise. Those of us who have searched for genes and molecules with specificity adequate to account for the observed biological phenomena have made progress but have not found the answers.

We now believe that we have identified some of the genes and molecules that provide the required very high specificity on cell surfaces and thus we have begun to uncover the genetic and molecular basis of the cell surface address codes indicated by the biological phenomena mentioned above.

The new genomic databases that have become available during the past few years have allowed us to carry out research that could not be done previously in our local laboratories. Accordingly, we have made extensive use of genomic techniques and the databases to obtain the new results. We feel that we have now benefited greatly from investments made in previous decades at Caltech to develop the microchemical instruments used in the genome projects.

Recently, we have begun to examine the possibility that genetic machinery, evolutionarily related to two types of genetic switches that are known to be used in the developing immune system, are also used in other developmental systems. We have written genomic software ("Tapestry") to aid in this project. In addition, experiments have been initiated in collaboration with Scott Fraser and David Koos in the Beckman Institute Image Center to explore the role of genetic switches in development.

299. Olfactant receptors have a dual function and may play a central role in cellular dressing for many types of organogenisis

William Dreyer

Studies in our laboratory as well as many others have succeeded in identifying a large number of cell surface molecules that play a role in cellular assembly and seem to function much like the country codes, area codes and prefixes of a telephone dialing system. However, the predicted highly specific final part of the code, equivalent to the last four digits of a unique phone number, has eluded us until very recently when we uncovered a new type of candidate code molecule: the odorant receptors. These serpentine receptors (they pass through the cell membrane seven times) are represented by thousands of genes in the human and mouse genomes. There are more than 1000 members of odorant receptor related families in *C. elegans* – more than the total number of cells in that organism. Recent research has shown that the odorant receptors not only detect odorants, a relatively common type of function for a protein molecule, but also play an extremely sophisticated role in axonal targeting as axonal processes extend from the olfactory epithelium to highly specific points (glomeruli) on topological maps on the olfactory bulb. Because these receptors are capable of such a high targeting specificity they bear the hallmarks of the proposed area code molecules that we believe aid in the assembly of most tissues. It therefore seemed appropriate to ask if they might be expressed in other parts of the developing embryo (and adult) as expected for such molecular codes. A search of the genome and literature databases revealed a remarkable number of examples of these genes expressed in tissues other than the olfactory system. The widespread expression in numerous organ systems of molecules related to the olfactory receptor family obviously supports the hypothesis that such receptors perform functions other than the recognition of olfactants. Since receptors of this type play a dual role as receptors for small molecules in the olfactory epithelium and as cell surface addressing molecules that aid in the assembly of the olfactory bulb of the brain, one obvious notion is that they may also play an assembly role in other parts of the brain and embryo and are the much-sought-after key cell surface molecules used in assembling embryonic tissues.

References

Dreyer, W.J. (1999) *Genetica* **107**:249-259.

Mombaerts, P., Wang, F., Dulac, C., Chao, S.K., Menes, A., Mendelsohn, M., Edmondson, J. and Axel, R. (1996) *Cell* **87**:675-686.

300. Homophilic interactions of olfactory receptors can explain otherwise perplexing experimental observations

William Dreyer

Scientists using the two-photon confocal microscopes in the Beckman Institute Biological Imaging Center (headed by Scott Fraser) have been able to observe

olfactory axons as their growth cones migrate to the brain (see references below). I have been fortunate to be able to observe this work and have used it, together with studies from other labs, to draw the following conclusions: 1) Axons that express one specific receptor have a strong tendency to fasciculate (bond) to themselves to the exclusion of the 1,000 or so axons that display other receptors; 2) As a specific axon homes in on its target glomerulus, the growth cones sometimes move to the correct target after first going near a neighboring glomerulus (see below re heterophilic interactions); 3) When all olfactory axons are labeled (with olfactory marker protein) it can be seen that thick bundles of axons emerge from each adult glomerulus without obvious mixing with other fascicles.

These observations argue strongly that each of the 1,000 or so olfactory receptors is capable of recognizing itself with highly specific homophilic interactions. Since these receptors are expressed on cells of essentially all tissue types, homophilic interactions would add high specificity to cell-cell interactions throughout the organism. While the proposed homophilic interactions seem highly likely based on the experimental evidence mentioned above, heterophilic interactions with very similar receptors on neighboring cells could provide a "gradient of receptor affinities," a potentially powerful mechanism for the assembly of organisms (see Dreyer, 1998; Dreyer and Roman Dreyer, 1999).

References

Dreyer, W.J. (1998) *Proc. Natl. Acad. Sci.* USA **95**:9072-9077.

Potter, S.M., Zheng, C., Koos, D.S., Feinstein, P., Fraser, S.E. and Mombaerts, P. (2001) *J. Neurosci.* **21**:9713-9723

Publications

Dreyer, W.J. and Roman-Dreyer, J. (2000) Mobile-element related gene switches generate precise and heritable cell-surface displays of address molecules that are used for constructing embryos. In: "Transposable Elements and Genome Evolution," (J.F. McDonald, ed.), Kluwer Academic Publishers, Dordrecht, Boston, London.

The Allen and Lenabelle Davis Professor of Biology: Mary B. Kennedy
Associate Biologist: Leslie T. Schenker
Assistant Biologist: Alan Rosenstein
Senior Postdoctoral Scholars: Pat Manzerra
Postdoctoral Scholars: Eugenia Khorosheva, Irene Knuesel, Vladan Lucic, Jeong Oh
Graduate Students: Stefan Mihalas[1], Luis Vazquez
Research and Laboratory Staff: Carene A. Oliveras-Garcia[1], Cheryl Gause, Shannon O'Dell
[1]*Graduate Student in Physics, Caltech*
[2]*Undergraduate, University of Puerto Rico*

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Summary: The Kennedy lab studies molecular mechanisms of synaptic regulation in the central nervous system. Memories are stored in the brain through long-lasting changes in the strength of synapses between neurons. These changes are triggered when the synapses are used to perceive the object or event being remembered. Regulation of synaptic strength may also underlie mood changes and is important for minute by minute information processing. Some neurotransmitters, such as glutamate and acetylcholine, which activate ligand-gated ion channels, can also initiate long-lasting biochemical changes that change synaptic strength. Excitatory synapses contain signaling protein complexes at the postsynaptic membrane, within a structure called the postsynaptic density (PSD). We are interested in the structure and functional organization of these signaling complexes and their roles in synaptic plasticity.

We have used cell biological, microchemical and molecular genetic methods to identify the protein components of the PSD fraction purified from brain (Kennedy, 1997, 2000). These include the scaffold molecule PSD-95; the NR2B subunit of the NMDA-type glutamate receptor; Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II); a Ras GTPase-activating protein called synGAP; a putative adhesion molecule, densin-180; a myosin motor termed myosin V or "dilute myosin" and a septin molecule, cdc10.

By now, most of the major proteins in the PSD fraction have been identified. In addition, sequencing and assembly of the human and mouse genomes are nearly finished, providing us with a complete "parts list" for signaling complexes. This is an exciting time, because we can turn our attention to the task of understanding how proteins are organized at the synapse and how they function together as a signaling machine.

One important synaptic signaling complex assembles around the cytosolic tail of the NMDA receptor. This complex includes many of the proteins that we found in the postsynaptic density. Activation of the NMDA receptor leads to influx of calcium and activation of CaM kinase II. It is well known that disruption of the CaMKII gene leads to derangement of synaptic regulation. CaMKII can phosphorylate the protein synGAP, and may regulate its location or activity at synapses, but the function of synGAP is unknown. We have recently used homologous recombination in embryonic stem cells to create mutant mice in which the synGAP protein is deleted, and are using the mutant mice to establish when and how the synGAP protein is functionally significant.

We found that a second signaling complex in the PSD forms around the tail of the PDZ-domain protein, densin-180. This high-affinity ternary complex contains CaMKII and the actin-binding protein α-actinin. Densin-180 is the founding member of a family of proteins now termed LAP proteins (LRR-domain and PDZ-domain containing proteins). The phenotypes of mutants in LAP proteins in *C. elegans* and *Drosophila* indicate that these proteins play important roles in directing the polarized localization of receptors and other proteins. We are using several strategies to gain insight into the functional role of densin-180 at the synapse.

In conjunction with our biochemical and cell biological experiments, we have taken a new direction in the lab and are using computer simulations and measurements of rapid kinetics to study the kinetics and interactions of signaling pathways in the tiny postsynaptic spine. The complex signaling machinery at the synapse integrates a variety of signaling influences and determines the "set-point" of synaptic strength at individual synapses. Many important signaling reactions are triggered by calcium influx into the postsynaptic spine, and result in phosphorylation of proteins, eventually leading to changes in synaptic strength. We have initiated a collaboration with scientists at the Salk Institute to build computer simulations of the flux of protein phosphorylation events in spines in the hippocampus. We will use the program MCell to implement stochastic simulation methods that model the position and behavior of immobilized signaling molecules within the spine. We will formulate our quantitative understanding of each protein and reaction in a signaling scheme, and assemble these into a complex kinetic simulation in MCell. We will then generate testable predictions about the flow of protein phosphorylation events in excitatory spines and dendrites, under conditions of calcium influx that cause changes in synaptic strength. Our ultimate goal is to create simulations that will illuminate our understanding of biochemical information coding at different types of synapses in the brain. Predictions arising from the simulations will be tested experimentally by measuring the time course and spatial distribution of phosphorylation of CaMKII and other phosphorylated synaptic molecules under a variety of physiological conditions. This effort will lay the groundwork for understanding the behavior of highly interconnected signaling pathways at the synapse.

References
Kennedy, M.B. (1997) *Trends Neurosci*. **20**:264-268.
Kennedy, M.B. (2000) *Science* **290**:750-754.

301. Structure of the densin gene

Eugenia Khorosheva

Our laboratory identified densin-180 (hereafter referred to as densin) in the postsynaptic density (PSD) fraction by microsequencing and cloning (1). Densin is expressed only in brain and is highly concentrated in the PSD at synapses. It includes a large, highly glycosylated extracellular domain, a single transmembrane domain, and a relatively short intracellular domain that ends in a PDZ domain. The amino terminus contains sixteen 23-residue leucine-rich repeats (LRRs). Proteins with a similar domain structure have been found to play a role in the functional polarization of various epithelial tissues. We found that the intracellular domain of densin forms a high-affinity ternary complex with CaM kinase II and the actin-associated protein α-actinin. Thus, densin may act as one docking site for CaM kinase II in the postsynaptic density. Our laboratory and others have found evidence for alternative splicing of densin transcripts. We set out to characterize the exon structure of the densin gene. We were able to find the sequence of the mouse gene encoding densin in a genomic database. By comparison to sequences of densin cDNAs, we determined that the mouse gene for densin is 356 kb in length and contains 27 exons. There appear to be at least seven splice variants. We are presently using this information to design strategies for determining densin's functions.

Reference

(1) Apperson, M., Moon, I.S. and Kennedy, M.B. (1996) *J. Neurosci*. **16**:6839-6852.

302. Effect of deletion of synGAP in two to five week old mice

Irene Knuesel

SynGAP is expressed throughout the brain in excitatory neurons and in a subpopulation of GABAergic neurons. Mice that are homozygous for a deletion of synGAP die a few days after birth. The cause of death is not yet clear, nor is it known what effect a deletion of synGAP would have on neural function in older mice. To investigate this issue we have created mice with a floxed synGAP gene so that the gene can be excised when CRE recombinase is expressed. We are mating mice carrying the floxed gene with mice engineered to express CRE recombinase driven by the CaM kinase II α-subunit promoter. This promoter drives expression of proteins, primarily in forebrain pyramidal neurons, beginning about day eight after birth and reaching a peak about day 14 after birth. The precise rate of disappearance of the synGAP protein will depend on the rate of its degradation. We are examining the content of synGAP in these mice at different ages in various brain areas and neuronal subtypes. When we have determined at what age the synGAP protein is depleted, we will study changes in neural function in the mice at that age.

303. A general kinetic model for activation of CaMKII at low concentrations of Ca^{2+} and calmodulin

Vladan Lucic, Stefan Mihalas

CaM kinase II (CaMKII) has been shown to be a crucial component of several important neural protein phosphorylation pathways. In particular, it is concentrated beneath the postsynaptic membrane of excitatory synapses in the cortex and hippocampus and is a major target of the calcium ion flowing through the activated NMDA receptor. In order to include it in simulations of signal transduction in the spine, we are constructing a kinetic scheme that we believe captures the essential features of activation of CaMKII by Ca^{2+} and calmodulin under conditions present in spines. Activation of CaMKII involves the binding of four Ca^{2+} ions to individual calmodulin molecules and the association of calmodulin with a specific site on each CaMKII subunit that leads to activation of the catalytic domain. The CaMKII holoenzyme is a dodecamer of individual catalytic subunits held together in two parallel rings of six by the association domains of each subunit. When calmodulin binds to two adjacent subunits in one of the rings, one of the two subunits is autophosphorylated at a specific site that causes the subunit to remain activated even after the Ca^{2+} concentration falls. When a subunit has been autophosphorylated, its activity ceases only when it is dephosphorylated by cellular phosphatases. Last year, we measured autophosphorylation of CaMKII at a variety of concentrations of Ca^{2+} and calmodulin similar to those we believe would be present in spines. This year, we constructed a general kinetic model of activation that fits this data reasonably well and is consistent with kinetic data already in the literature. We uncovered interesting features of activation of CaMKII that were not previously appreciated. For example, the data are only fit well if one assumes that calmodulin with less than four Ca^{2+} ions can bind to CaMKII and that after this event, binding of the final Ca^{2+} ions is highly accelerated. We believe our findings will help to clarify very early events involved in activation of CaMKII in spines.

304. Signal transduction in SynGAP mutant mice

Pat Manzerra

SynGAP is a synaptic ras GTPase-activating (GAP) protein identified by us in the postsynaptic density fraction prepared from rat forebrain (see Abstract 307). We have hypothesized that, by catalyzing inactivation of Ras, synGAP down regulates signaling through various growth factor receptors at synapses. To gain more insight into the functions of synGAP *in vivo*, our lab used homologous recombination to generate mice in which the gene encoding synGAP is deleted. Although the homozygous mutant mice die a few days after birth, primary neuronal cultures prepared from cortex and hippocampus of the mutants live in culture as long as

wild-type neurons (three to four weeks). We are comparing signal transduction through the NMDA receptor and through various growth factor receptors in the neuronal cultures from WT and mutant mice. In addition, there is mounting evidence that the Ras-activated MAP kinase pathways might be involved in mediating cell death under pathological conditions, including ischemia due to stroke, and seizure. Therefore, we are also investigating the possibility of an effect of the synGAP mutation in *in vitro* models of pathological neuronal cell death.

305. Measurement of rapid kinetics of activation of CaMKII at low concentrations of Ca^{2+} and calmodulin

Stefan Mihalas, Mary Kennedy

As described in (Abstract 303), we are constructing a kinetic scheme for activation of CaM kinase II by Ca^{2+} and calmodulin under conditions present in spines. In order to test predictions of possible models, and to determine certain crucial kinetic constants for the models, we have begun to measure the rapid kinetics of activation of CaM kinase II at varying low concentrations of Ca^{2+} and calmodulin. To do this we are employing a rapid quench-flow apparatus that will mix two solutions at controlled temperature to begin the kinase reaction, then quench the reaction with SDS-sample buffer after a time period that can vary from a few milliseconds to a few minutes. The samples are then analyzed for activation of CaM kinase II by quantitative immunoblotting with a phosphosite-specific antibody against the autophosphorylation site of CaM kinase II. Activation of CaM kinase II involves the binding of four Ca^{2+} ions to individual calmodulin molecules and the association of calmodulin with the calmodulin-binding domain of each CaM kinase II subunit. When calmodulin binds to two adjacent subunits in one of the six-membered rings in a CaM kinase II holoenzyme, one of the two subunits is autophosphorylated at a specific site, which causes the subunit to remain activated even after the Ca^{2+} concentration falls. This sequence of events leads to complex kinetics of activation. The extent of cooperativity of binding of the four Ca^{2+} ions to calmodulin in the presence of CaM kinase II is a crucial variable determining the extent of CaM kinase activation at low Ca^{2+} concentrations. The comparison of the rapid kinetic measurements to time courses of reaction predicted by various models will help us to deduce values for crucial parameters to be used in building simulations of signal transduction in spines.

306. Phosphorylation of synGAP by CaM kinase II does not inhibit its activity

Jeong Oh

Our lab previously reported that phosphorylation of synGAP by CaM kinase II inhibits its GTPase-activating activity (1). This year we found that synGAP activity is, in fact, not directly inhibited by phosphorylation by CaM kinase II. The experiments presented in the previous paper involved two successive enzymatic reactions. In the first, synGAP in the postsynaptic density was prephosphorylated by CaM kinase II in the presence of Ca^{2+}, calmodulin, and 0.1 mM ATP. Phosphorylated PSD fractions were then assayed for ras GTPase activating activity (GAP activity). Pyrophosphate, a potent inhibitor of phosphatases in the PSD fraction, was added to the GAP assay to preserve the phosphorylation state of synGAP. Because of a fault in the design of the experiments, controls were not included in which *both* ATP and pyrophosphate were present in GAP assays containing nonphosphorylated synGAP. We have now found that the combination of residual ATP (30 μM) from the prephosphorylation reaction and 6 mM sodium pyrophosphate is sufficient to inhibit the GAP activity of synGAP. No additional inhibition is produced by phosphorylation of synGAP by CaM kinase II. The error was reinforced by an experiment shown in the paper that indicated inhibition of synGAP activity was blocked when inhibiting antibodies against CaM kinase II were included during the prephosphorylation reaction. We cannot reproduce blockade of synGAP inhibition by these antibodies. We published a correction (2) and indicated that the paper would be more appropriately titled "A Synaptic Ras-GTPase Activating Protein Phosphorylated by CaM Kinase II."

References

1. Chen, H-J., Rojas-Soto, M., Oguni, A. and Kennedy, M.B. (1998) *Neuron* **20**:895-904.
2. Oh, J.S., Chen, H-J., Rojas-Soto, M., Oguni, A. and Kennedy, M.B. (2002) *Neuron* **33**:151.

307. Phosphorylation sites for CaM kinase II on synGAP

Jeong Oh, Alan Rosenstein, Pat Manzerra

Our lab has shown that SynGAP is a prominent target in the postsynaptic density for phosphorylation by CaM kinase II. In order to study the physiological effects of this phosphorylation, we have identified individual phosphorylation sites in the synGAP sequence. SynGAP was exhaustively phosphorylated in the presence of γ-^{32}P-labeled ATP. After separation of the labeled protein by SDS-polyacrylamide gel electrophoresis the protein was isolated and subjected to trypsinization within the gel piece. Individual ^{32}P-labeled peptides were purified by high-pressure liquid chromatography and their sequences determined by mass spectrometry or gas-phase Edman degradation with help from the Caltech Protein and Peptide Microanalysis Lab. We found two major phosphorylation sites that are phosphorylated rapidly and to high stoichiometry by CaM kinase II. These same sites are also phosphorylated by CaM kinase II in the postsynaptic density fraction. We attempted to raise phospho-site specific antibodies against these sites in rabbits, and were successful at doing so for one of the sites. We used the affinity purified phospho-site specific antibody to show that the site is phosphorylated in hippocampal neurons in culture. We are presently

constructing mutations in these sites to help us determine the regulatory effect of their phosphorylation.

308. The role of the intracellular domain of the NMDA-type glutamate receptor

Leslie Schenker, Rolf Sprengel, Peter Seeburg*, Mary Kennedy*

The NMDA-type glutamate receptor in the central nervous system is composed of NR1 subunits of approximately 110 kDal and combinations of four NR2 subunits of approximately 160 to 180 kDal. The larger size of the NR2 subunits reflects the presence of carboxyl terminal tails of about 300 amino acids that extend into the cytosol. The cytosolic tails of the NR2 subunits associate with intracellular signaling machinery that regulates the strength of synaptic transmission. The principal NR2 subunits in the hippocampus and cerebral cortex are NR2A and NR2B. Mutant mice lacking the intracellular C-terminal tails of the NR2A (NR2ADC) and NR2B (NR2BDC) subunits were created in the Seeburg lab to study the role of these domains in receptor function *in vivo* (1). NR2BDC mice die shortly after birth, whereas NR2ADC mice are viable, but show defects in regulation of synaptic transmission. We are breeding mice that produce embryos lacking the tails of both NR2A and NR2B subunits. We can culture dissociated neurons from the brains of these embryos. Neurons cultured from double truncation mutants show marked defects in transport of specific proteins to the synapse. We are now studying the implications of these defects for mechanisms of trafficking of synaptic proteins.

**Max Planck Institute for Medical Research, Heidelberg, Germany*

Reference

(1) Sprengel, R. *et al.* (1998) *Cell* **92**:279-289.

309. Synapse formation in SynGAP knockout mice

Luis E. Vazquez, Carene A. Oliveras

SynGAP is a brain-specific, Ras GTPase activating protein (GAP), located in the postsynaptic density of glutamatergic synapses. In addition to its GAP domain, it contains PH and C2 domains, a proline-rich region, and a t-SXV motif that interacts with the scaffold protein PSD-95. It is a prominent component of the postsynaptic density (PSD) and forms part of the NMDA receptor signaling complex. To study the role of synGAP in neurons, we generated a synGAP knockout mouse by deleting several exons from the synGAP gene. Mice homozygous for this mutation do not express any detectable synGAP protein. These mice fail to thrive, and die two to five days after birth. To study synapse formation in these mutants, we dissociated and cultured embryonic hippocampal neurons at day E15-16. We find that in the cultures, spine formation and maturation is significantly accelerated in neurons from mutant mice compared to neurons from their wild type litter mates. Thus, by ten days *in vitro*, the mutant neurons have a greater density of spiny protrusions compared to wild type. Clusters of PSD-95 are observed at the tips of most of the spines in the mutant, whereas in wild-type neurons of this age, they are still located in the dendritic shaft. Also at ten days *in vitro*, twice the proportion of spines in mutant neurons contain both AMPA- and NMDA-receptors, compared to wild-type neurons. By twenty-one days *in vitro*, the mutant neurons generally have fewer postsynaptic terminals than wild type, but they are larger in size and stain more brightly for various synaptic proteins. These data suggest that synGAP plays an important role in regulating the assembly and organization of spine synapses.

Publications

Kennedy, M.B. (2001) Beautiful transfer of information. *Science* **293**:2210-2211.

Kennedy, M.B. (2002) Calcium/Calmodulin-Dependent Protein Kinase II. In: *Handbook of Cellular Signaling*. In press.

Kennedy, M.B. (2002) The NMDA-Receptor Signaling Complex. In: *Handbook of Cellular Signaling*. In press.

Kennedy, M.B. and Manzerra, P. (2001) Telling tails. *Proc. Natl. Acad. Sci. U.S.A*. **98**:12323-12324.

Oh, Jeong S., Chen H.-J., Rojas-Soto, M., Oguni, A., and Kennedy, M.B. (2002) Erratum. *Neuron* **33**:151.

Vazquez, L.E., Chen, H.-J. Oliveras, C.A., Kennedy, M.B. (2002) Synapse formation in synGAP knockout mice. *Soc. for Neurosci. Abstr*. **28:**746.5.

Bren Professor of Biology: Henry A. Lester
Norman Chandler Professor Emeritus of Chemical Biology: Norman Davidson
George Grant Hoag Professor of Chemistry: Dennis Dougherty[1]
Visiting Associate: Johannes Schwarz
Members of the Professional Staff: Bruce Cohen, Cesar Labarca
Senior Postdoctoral Fellow: Chi-Sung Chiu
Postdoctoral Fellows: David Dahan[1], Mohammed Dibas, Carlos Fonck, Abraham Kovoor, John Leite, Ming Li, Ping Li, Fraser Moss, Alexa Mundy, Raad Nashmi, Irina Sokolova, Andrew Tapper, Tzu-Ping Yu
Graduate Students: Darren Beene[1], Gabriel Brandt[1], Amanda Cashin[1], Don Elmore[1], Lintong Li[1], Joshua Maurer[1], Sarah Monahan[1], Tingwei Mu[1], James Petersson[1], Nivalda Pinguet[2], George Shapovalov[3], Eric Slimko, Steve Spronk[1], Niki Zacharias[1]
Rotating Graduate Students: Julien Muffat, Amber Southwell
SURF Students: Vincent Auyeung, Andrew Dervan, Kai Sung, Lisa Turner, Jialan Wang
Associate Biologist: Purnima Deshpande
Research and Laboratory Staff: Matt Abramian, Sami Barghshoon, Shannan Boss, Pamela Fong, Donghong Ju, Kira Kostenko, Sheri McKinney, Giovanna Santoro, Carrie Shilyansky, Yuping Wang

[1]*Division of Chemistry and Chemical Engineering, California Institute of Technology*
[2]*Special Graduate Student, Division of Biology, California Institute of Technology; Graduate Student, University of California, Riverside*
[3]*Division of Physics, Mathematics and Astronomy, California Institute of Technology*

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Summary: We continue our work on ion channels, receptors, and transporters. We have continued to analyze several strains of knock-in mice generated in our laboratory for two ligand-gated channels, the nicotinic α4 receptor and the serotonin 5-HT3 receptor. The former has generated interesting insights into neurodegenerative disease and epilepsy. The latter has generated insights into murine urologic syndrome.

Our work on selective silencing of mammalian neurons has generated a promising set of techniques and reagents based on ligand-activated chloride channels. We are now generating "proof of concept" transgenic mouse strains.

We continue our joint work with the Dougherty group, in Caltech's Chemistry Division, on aspects of ion channel structure-function. We have brought novel techniques to these studies, including mass spectrometry and fluorescence. We work on unnatural amino-acid mutagenesis and attempt to extend this method to mammalian cells. We collaborate with both Dougherty and Doug Rees, also in the Chemistry Division, on bacterial ion channels of known atomic-scale structure.

Our work continues on quantitative aspects of transporter function, primarily measured with fluorescence and with knock-in mice. As an interesting side benefit of the GABA transporter knock-in mouse, we have generated and analyzed a knockout mouse for the same molecule.

The late Norman Davidson led a subgroup working on aspects of synaptic plasticity, particularly those that depend on A kinase stimulation. Members of this subgroup are now analyzing their data and preparing papers for publication.

Our group's home page has additional up-to-date information, images, and notices of positions. It's at http://www.its.caltech.edu/~lester.

310. Increased sensitivity to agonist-induced seizures and analgesia in knock-in mice carrying hypersensitive α4 nicotinic receptors

Carlos Fonck, Raad Nashmi, Purnima Deshpande, M. Imad Damaj[1], Michael J. Marks[2], Johannes Schwarz, Allan C. Collins[2], Cesar Labarca

We studied a strain of exon replacement mice ("L9'S knock-in") whose α4 nicotinic receptor subunits possess a leucine to serine mutation in the M2 region, 9' position [Labarca *et al*. (2001) *PNAS*], this mutation renders α4-containing receptors hypersensitive to agonists. [^{125}I]Epibatidine binding on brain membranes showed that the mutant allele was expressed at ~25% of WT levels, presumably because of a neomycin selection cassette in a nearby intron. ^{86}Rb efflux experiments on brain synaptosomes showed an increased fraction of function at low agonist concentrations in L9'S mice. Nicotine-induced seizures at concentrations ~ 8 times lower in L9'S (1 mg/kg) than in WT littermates. At these concentrations, L9'S, but not WT, showed increases in EEG amplitude and theta rhythm. L9'S mice also showed higher seizure sensitivity to the nicotinic agonist epibatidine, but not to the $GABA_A$ receptor blocker and proconvulsant bicuculline. Dorsiflexion of the tail (Straub tail) was the most sensitive nicotine effect found in L9'S mice (0.1 mg/kg). Galanthamine and tacrine produced similar seizures and Straub tails in the L9'S mice, arguing across the idea that these two drugs have different actions. Nicotine caused greater analgesia in L9'S than in WT mice in the hot plate nociception assay, but not in the tail flick assay. No apparent neuroanatomical differences were found in anti-α4 and Nissl-stained hippocampal sections from L9'S and WT mice. These data support: (1) the importance of the α4 subunit in mediating nicotine analgesia in supraspinal responses, but not primarily spinal reflex-dominated pathways; and (2) the possible involvement of gain-of-function α4 receptors in autosomal dominant nocturnal frontal-lobe epilepsy.

[1]*Department of Pharmacology and Toxicology, Medical College of Virginia/VCU, Richmond VA 23298*

[2]*Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309*

311. Mutant mice carrying hypersensitive α4 nicotinic receptors

Cesar Labarca, Purnima Deshpande

We have previously generated knock-in mice with a Leu9'Ser mutation in the M2 region of the α4 subunit of the neuronal acetylcholine receptor (AChR). This mutation give the α4β2 AChR higher sensitivity to agonist which results in a gain of function of the receptor. Four genotypes can be produced, depending on the number of mutated alleles and the presence of the neomycin cassette, which markedly reduces the expression of the mutated gene. The corresponding phenotypes differ in the magnitude of the gain of function. Only the L9'S mice with the mildest gain of function phenotype, heterozygous for the mutation and containing the neomycin cassette, survive beyond the first day and reproduce normally.

We have generated now knock-in mice with a Leu9'Ala mutation, in an effort to get a broader range of useful genotypes. This mutation produces a milder gain of function, as tested in oocytes (about 7-fold) than the Leu9'Ser mutation (about 35-fold). All four genotypes (heterozygous neo-intact and neo-deleted, and homozygous neo-intact and neo-deleted) survive and reproduce normally. Preliminary experiments with a limited number of animals to determine their sensitivity to nicotine indicate that the phenotypes range from very mild, in the heterozygous neo-intact, to a much stronger phenotype in the homozygous mice.

312. Design and analysis of ADNFLE mutant S10'L α4 nAChR knock-in mice

Andrew R. Tapper, Purnima Deshpande, Cesar Labarca

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is an idiopathic epileptic disorder characterized by nocturnal seizures localized within the frontal lobe arising during stage 2 sleep. Mutations within the putative pore-lining M2 helix of ionotropic neuronal nicotinic acetylcholine receptor α4 (CHRNA4) and β2 (CHRNAB2) subunits have been linked to ADNFLE. When expressed in heterologous expression systems, ADNFLE-associated mutant receptors have altered channel properties compared to wild type, suggesting that malfunctioning nicotinic receptors are responsible for the disease phenotype. Despite the molecular identification and electrophysiological characterization of nAChR mutations that may underlie some cases of ADNFLE, many questions remain regarding the pathophysiology of the disease. For example: Why are seizures localized to the frontal lobe of the neocortex? Why do seizures arise only during sleep? In general, what is the mechanism by which mutant acetylcholine receptors induce epileptiform activity? To address these questions we are in the process of genetically engineering an ADNFLE mutant (S10'L) nAChR α4 knock-in mouse as an *in vivo* model for ADNFLE. To date, we have designed a S10'L knock-in construct, introduced the linearized construct into mouse embryonic stem (ES) cells via electroporation, and screened for recombinants using a combination of G418 resistance and PCR. In addition, sequence analysis has been used to confirm the presence of the mutation in each positive ES clone. Two of the positive clones have been electroporated with the cytomegalovirus (CMV) Cre plasmid. Successful transfection with the CMV-Cre plasmid will lead to deletion of the neomycin cassette, thereby preventing Neo cassette-induced lower gene expression. We are now in the process of screening these clones for the absence of the Neo cassette by analyzing their susceptibility to G418. Once identified, the Neo-deleted S10'L ES clone will be injected into C57BL/6 blastocysts and implanted into a pseudopregnant C57BL/6 female mouse.

Generation of the S10'L line, followed by behavioral and electrophysiological characterization of the mutant mice, should provide insight into the pathogenic mechanism and pathophysiology of an epileptic disorder, as well as contribute to a better understanding of acetylcholine receptor function in the cortex.

313. A single mutation in the α4 amino-terminal domain eliminates Ca^{2+} potentiation of the α4β2 nicotinic acetylcholine response

Nivalda Rodrigues-Pinguet, Li Jia[1], Bruce Cohen

Previous experiments show that a point mutation (E172Q) in the amino (N)-terminal domain of the α7 neuronal nicotinic acetylcholine receptor (nAChR) eliminates potentiation of the acetylcholine (ACh) response by extracellular Ca^{2+} [Galzi *et al*. (1996) *EMBO J.* **15**:5824-5832]. The glutamate at position 172 in α7 is highly conserved among nAChR subunits and lies inside a loop that binds Ca^{2+} ions in the snail ACh-binding protein. To determine whether the homologous residue in α4 & β2 plays an essential role in Ca^{2+} potentiation of the rat α4β2 ACh response, we mutated the corresponding glutamate to glutamine (α4(E180Q) and β2(E177Q), co-expressed the mutated subunits with the appropriate WT subunits in *Xenopus* oocytes, and tested their effects on Ca^{2+} potentiation at two ACh concentrations (30, 1000 μM). The α4(E180Q) mutation eliminated 2 mM Ca^{2+} potentiation of the α4β2 response at both ACh concentrations. At 30 μM ACh, the ratio of the peak ACh response with 2 mM added extracellular Ca^{2+} to that with no added Ca^{2+} (I_{Ca}^{2+}/I_0) was 5 ± 1 (mean ± SEM, n = 3 oocytes) for the WT and 0.85 ± 0.05 (n = 7) for the α4(E180Q) mutant. Similarly at 1000 μM ACh, the I_{Ca}^{2+}/I_0 was 6 ± 1 (n = 2) for the WT and 1.10 ± 0.03 (n = 4) for the mutant. Moreover, the α4 (E180Q) mutation did not affect receptor surface expression or the ACh concentration-response relationship but it did reduce the maximum ACh response. In contrast to the α4(E180Q) mutation, the β2(E177Q) mutation did not affect calcium potentiation of the α4β2 ACh response. However, it did shift the ACh concentration response dramatically to the right. Thus, a conserved glutamate at position 180 in the N-terminal domain of α4 plays an essential role in Ca^{2+} potentiation of the α4β2 ACh response.

[1]*Division of Biomedical Sciences, University of California, Riverside, CA*

Reference
Galzi, J.L., Bertrand, S., Corringer, J.P., Changeux, J.P. and Bertrand, D. (1996) *EMBO J.* **15**:5824-5832.

314. α4β2 neuronal acetylcholine receptors: Targets for both nicotine and ethanol

Jeremy C. Owens[1], Seth A. Balogh[1], Tristan D. McClure-Begley[1], Cesar Labarca, Marina R. Picciotto[2], Jeanne M. Wehner[1], Allan C. Collins[1]

Recent evidence argues that common genes influence alcohol and tobacco abuse in humans. This finding prompted us to search for common genes that regulate behavioral responses to ethanol and nicotine in the mouse. The results of the studies reported here, which used three different genetic strategies, indicate that sensitivity to both nicotine- and ethanol-induced depression of acoustic startle is modulated by α4β2 neuronal nicotinic cholinergic receptors (nAChRs).

[1]*Institute for Behavioral Genetics, University of Colorado, Boulder, CO 80309*
[2]*Department of Psychiatry, Yale University, New Haven, CT, 06508*

315. Selective electrical silencing of mammalian neurons *in vitro* using invertebrate ligand-gated chloride channels

Eric M. Slimko, Sheri McKinney, David J. Anderson[1]

Selectively reducing the excitability of specific neurons will: (1) allow for the creation of animal models of human neurological disorders; and (2) provide insight into the global function of specific sets of neurons. We focus on a combined genetic and pharmacological approach to silence neurons electrically. We express invertebrate ivermectin (IVM)-sensitive chloride channels (*C. elegans* GluCl α and β) with a Sindbis virus, then activate these channels with IVM to produce inhibition via a Cl^- conductance. We constructed a three-cistron Sindbis virus that expresses the α and β subunits of GluCl along with the green fluorescent protein (EGFP) marker. Expression of the *C. elegans* channel does not affect the normal spike activity or GABA/glutamate postsynaptic currents of cultured E18 hippocampal neurons. At concentrations as low as 5 nM, IVM activates a Cl^- current large enough to effectively silence infected neurons. This conductance reverses in eight hours. These low concentrations of IVM do not potentiate GABA responses. Comparable results are observed with plasmid transfection of yellow fluorescent protein (EYFP) tagged GluCl α and cyan fluorescent protein (ECFP) tagged GluCl β. The present study provides an *in vitro* model mimicking conditions that can be obtained in transgenic mice and in viral-mediated gene therapy. These experiments demonstrate the feasibility of using invertebrate ligand-activated Cl^- channels as an approach to modulate excitability.

[1]*Professor, Division of Biology*

316. Selective elimination of glutamate activation and introduction of fluorescent proteins into a *C. elegans* chloride channel

Ping Li, Eric M. Slimko

Glutamate-gated (GluCl) chloride channels from invertebrates can be activated by ivermectin (IVM) to produce electrical silencing in mammalian neurons. To improve this GluCl/IVM strategy, we sought to mutate the *C. elegans* GluCl channels so that they become insensitive to glutamate but retain their sensitivity to IVM. Based on structure-function studies of nAChR superfamily members, we tested in oocytes 19-point mutants at 16 residues in the β subunit likely to be involved in the response to glutamate. Y182F reduces the glutamate response by greater than 6 fold, with little change to IVM responses, when coexpressed with WT GluCl α. For GluCl αβ(Y182F), the EC_{50} and Hill coefficient for glutamate is similar to those of WT, indicating that the mutant decreases the efficacy of glutamate, but not the potency. Also, fluorescent proteins (EGFP, EYFP, ECFP; XFP) were inserted into the M3-M4 loop of the GluCl α, β and β(Y182F). We found no significant functional difference between these XFP-tagged receptors and WT receptors. The modified GluCl channel, without glutamate sensitivity but with a fluorescent tag, may be more useful in GluCl silencing strategies.

317. Codon optimization of *C. elegans* GluCl ion channel genes for mammalian cells significantly improves expression levels

Eric M. Slimko

Organisms use synonymous codons in a highly non-random fashion. This codon usage bias has been implicated in difficulties when expressing exogenous genes at high levels from widely divergent species. The *C. elegans* GluCl α1 and GluCl β genes form a functional glutamate and Ivermectin-gated chloride channel when expressed in oocytes, and can be weakly expressed in mammalian cells. We have constructed synthetic genes that retain the amino acid sequence of the wild-type proteins, but use codons that are optimal for mammalian cell expression. We have tagged both the native-codon-usage GluCl α1 gene and the codon-optimized GluCl α1 gene with Enhanced Yellow Fluorescent Protein (EYFP), and used fluorescence microscopy to measure the relative expression levels of the two genes. We have done a similar experiment with Enhanced Cyan Fluorescent Protein (EFCP)-tagged GluCl β genes. We verify our fluorescent measurement with electrophysiological recordings of these genes expressed in E18 rat hippocampal neurons. Codon optimization provides a six-to nine-fold increase in expression. The resulting Ivermectin-gated channel has an EC50 of 1.3 nM and a Hill coefficient of 1.9. Additionally, we have made the previously described Y182F mutation to the codon optimized β subunit, and find that while the Ivermectin-induced conductance remains the same, the glutamate-induced currents are seven-fold less. The final engineered channel should prove valuable in selectively silencing specific neuronal populations *in vivo*.

318. Characterization of α4β2 nicotinic receptor-fluorescent protein chimeras

R. Nashmi, M.E. Dickinson[1], S. McKinney, C. Labarca, S.E. Fraser[2]

Although the mechanisms of nicotine addiction remain unclear, altered trafficking of neuronal nicotinic receptors (nAChR) may be one contributing mechanism that modulates neuronal excitability. To study such mechanisms, we constructed several fluorescently-tagged nAChR proteins, including yellow fluorescent protein (YFP) fused to the N-terminus (N) and intracellular loop (M3-M4) of α4 and cyan fluorescent protein (CFP) fused to the C-terminus (C) and M3-M4 loop of β2. In HEK293T cells, N-α4-YFP (41 ± 13 pA) and C-β2-CFP (81 ± 15 pA) had significantly reduced current amplitude with 300 μM ACh as compared to wild-type (WT) α4 β2 (396 ± 86 pA). M3-M4 α4-YFP showed only modest reduction of current (206 ± 62 pA), while M3-M4 β2-CFP responded at WT or greater levels (613 ± 169 pA). Fura-2 imaging of HEK293T cells showed increased intracellular calcium accumulation in WT, M3-M4 α4-YFP and β2-CFP constructs with 300 μM ACh, while N-α4-YFP and C-β2-CFP showed no detectable change in intracellular calcium. The M3-M4 α4-YFP and β2-CFP constructs, when transfected into midbrain neurons, were excluded from the nucleus and were distributed in the soma and dendrites. Electrophysiology demonstrated the presence of functional surface α4-YFP β2-CFP. Fluorescence resonance energy transfer confirmed the association between α4-YFP and β2-CFP subunits: when YFP was bleached, CFP was dequenched. In conclusion, fusing a fluorescent protein in the M3-M4 loop of α4 or β2 showed less perturbation in function than those inserted at the N- or C-terminus.

[1]*Division of Biology*
[2]*Professor, Division of Biology*

319. Identification of nicotinic acetylcholine receptor α1 gating-dependent molecular motions using electrophysiology-coordinated photochemistry and mass spectrometry

John F. Leite, Michael P. Blanton[1], Mona Shahgholi[2], Dennis A. Dougherty

This study characterizes conformational changes of the muscle nicotinic acetylcholine receptor (nAChR) in the resting, open and desensitized states. We introduce electrophysiology-coordinated photolabeling (ECP) on *Xenopus* oocytes: receptors are photolabeled while their states are controlled on a millisecond time scale. Mass spectrometry then provides a rapid and sensitive method of detecting and identifying labeled residues.

Probes employed are benzophenone (BP), the acetyl- and trimethyl-acetyl- analogs of 3-trifluoromethyl-3-m-iodophenyl diazirine (TID), and a photoreactive cholesterol derivative. Because these probes reside within the phospholipid membrane, secondary structure information of domains at the protein-lipid interface can be inferred from the periodicity of photolabeled amino-acid residues.

Analysis of photolabeled nAChRα1 (resting state) proteolytic fragments identifies sites of probe incorporation within the fourth transmembrane domain (M4), as well as within the cytoplasmic loop (M3-M4), and within the conserved "signature loop" (residues 128-142) in the N-terminal domain. Photoincorporation into the "signature loop" agrees with the crystal structure of the acetylcholine binding protein, which suggests that this disulfide loop is located near the membrane surface.

Most importantly, we identify residues that are specifically labeled during the open state within the N-terminal domain. We propose several refinements to the present topological model for the acetylcholine receptor, and we suggest that elements within the amino-terminal domain as well as in the M3-M4 loop are involved in gating-dependent conformational shifts.

[1]*Dept. of Pharmacology, Texas Tech University Health Sciences Center*
[2]*Division of Chemistry and Chemical Engineering*

320. Spectroscopy reveals conformational changes coupled to nicotinic acetylcholine receptor gating

David S. Dahan, Dennis A. Dougherty, Henry Lester

The nicotinic acetylcholine receptor is a cation-selective channel that cycles between resting, open and desensitized states. The detailed mechanisms underlying these structural transitions remain unknown. Mouse muscle nAChR containing an M2 Cys mutation was expressed in *Xenopus* oocytes and treated with the sulfhydryl-reactive dye, methanethiosulfonate sulforhodamine (MTSR). MTSR modification occurred only in the presence of ACh and resulted in fluorescence signals that were approximately two-fold greater than those seen from control oocytes expressing wild-type nAChR. In time-resolved fluorescence experiments with labeled, voltage-clamped oocytes expressing the mutant nAChR, perfusion with ACh resulted in a 10-20% increase in fluorescence intensity. No such fluorescence intensity changes were seen with labeled oocytes expressing wild-type nAChR. These data implicate the channel-lining M2 domain in the conformational changes involved in nAChR gating.

321. Toward X-ray crystallography analysis of the extracellular domain (ECD) of the nicotinic α7 receptors

Mohammed I. Dibas, *Douglas C. Rees[1]*

Nicotinic receptors, a member of the ligand-gated superfamily, play a key role in the neurotransmission throughout the central and peripheral nervous systems. Agonist binding to a specific site in the ECD is the first step in a process that leads to channel opening. Therefore, exploring the atomic-scale details of the acetylcholine (an agonist) binding and its induced conformational change upon binding is a fundamental step toward drug development. Recent X-ray structural studies have determined the three dimensionality of the AChBP, which is homologous to the N-terminus of the nicotinic receptors, which encompass the agonist-binding domain. We have generated chimeras between the AChBP and the neuronal α7 ECD to determine which regions are responsible for the enhanced solubility and expression level of the AChBP in *Pichia pastoris*. We would like to

produce a soluble construct of the neuronal nicotinic acetylcholine α7 ECD in large amounts that are suitable for X-crystallography studies. The specific aims of our studies are 1) to test the hypothesis that the use of chimeras enhances the expression of the human α7 ECD in the *Pichia pastoris*; 2) to test the hypothesis that the ECD of the nicotinic α7 expressed in the *P. pastoris* has native-like pharmacological and biophysical properties; and finally 3) to characterize the transitions among native agonist-induced conformational states using X-ray crystallography.

[1]*Division of Chemistry and Chemical Engineering*

Reference

Brejc, K., J. van Dijk, W., Klaassen, R., Schuurmans, M., Van der Oost, J., Smit, A.B., Sixma, T.K. (2001). *Nature* **411**:269-276.

322. Cation-π interactions in ligand recognition by serotonergic (5-HT3A) and nicotinic acetylcholine receptors: The anomalous binding properties of nicotine

Darren L. Beene, Gabriel S. Brandt, Wenge Zhong, Niki M. Zacharias, Dennis A. Dougherty

A series of tryptophan analogues has been introduced into the binding site regions of two ion channels, the ligand-gated nicotinic acetylcholine and serotonin 5-HT3A receptors, using unnatural amino acid mutagenesis and heterologous expression in *Xenopus* oocytes. A cation-π interaction between serotonin and Trp183 of the serotonin channel 5-HT3AR is identified for the first time, precisely locating the ligand-binding site of this receptor. The energetic contribution of the observed cation-π interaction between a tryptophan and the primary ammonium ion of serotonin is estimated to be approximately 4 kcal/mol, while the comparable interaction with the quaternary ammonium of acetylcholine is approximately 2 kcal/mol. The binding mode of nicotine to the nicotinic receptor of mouse muscle is examined by the same technique and found to differ significantly from that of the natural agonist, acetylcholine.

323. An intersubunit hydrogen bond in nicotinic receptor function

Lintong Li, Dennis Dougherty

In May 2001, the crystal structure of acetylcholine binding protein (AChBP), that is homologous to the N-terminal extracellular domain of the nicotinic acetylcholine receptor (nAChR), was solved. Based on the model obtained from docking acetylcholine into the structure of AChBP, we designed and incorporated a new tethered agonist, lysyl-carbamylcholine. Incorporation of this tethered agonist at several positions produced constitutively active receptors, with significant activity seen at α192, α193, and γ119/δ121. These results demonstrated that the loop E residue γ119/δ121 on the complementary subunit is very near the agonist-binding site. We also investigated the role of an intersubunit hydrogen bond, which was seen in the crystal structure of AChBP. Incorporation of tryptophan analogs that abolish the hydrogen bonding abilities slowed the desensitization of the receptor, which implied that AChBP structure corresponded to the desensitized state of the nAChR. Our results have confirmed that AChBP is relevant to nAChR in general, and the desensitized state in particular.

324. Identification of a perturbed pk_a at the binding site of the nicotinic acetylcholine receptor through unnatural amino acid mutagenesis: Implications for nicotine binding

E. James Petersson, Anita Choi[1], Dennis A. Dougherty

A series of tethered quaternary ammonium derivatives of Tyr have been incorporated into the binding site of the nicotinic acetylcholine receptor (nAChR) using the *in vivo* nonsense suppression method, producing constitutively active (self-gating) receptors. We have incorporated primary, secondary, and tertiary amine tethered agonists to give receptors whose constitutive activity can be modulated by pH. Lowering the pH protonates the tethered amine, giving it a positive charge and allowing it to reversibly activate the receptor. Tertiary and secondary tethered amines, TyrO3T and TyrO3S, have been successfully incorporated at α149 in the nAChR. Constitutive currents at pH 5.5 are six times those at pH 9.0. The pK_a of TyrO3T in the binding site appears to be six or lower, differing substantially from its pK_a in solution (~9.3). This local pK_a perturbation has substantial implications for pharmacological research on the nAChR: of the tertiary agonists considered, noracetylcholine experiences this pK_a perturbation while nicotine does not.

[1]*Division of Chemistry and Chemical Engineering*

325. RGS9 via its DEP domain targets to D2 dopamine receptors and mice lacking RGS9 develop dyskinesia associated with dopamine pathways

Abraham Kovoor, Johannes Schwarz

We discovered that RGS9-2, a member of the RGS family of Gα GTPase accelerating proteins, associates with the D2-dopamine receptor (D2-DR), and preferentially accelerates the termination of D2-DR signals. We established that the DEP domain of RGS9 was both necessary and sufficient for association with D2-DR. DEP domains present in other proteins, such as disheveled, associate with G-protein coupled receptors (GPCRs) in addition to D2-DR suggesting that the DEP domain is a GPCR targeting domain. RGS9-2 is expressed specifically in the striatum, the brain region involved in the development of neuroleptic-induced tardive dyskinesia and levodopa-induced dyskinesia. We produced similar disorders in RGS9 knock-out mice when inhibition of dopaminergic transmission was followed by activation of D2 dopamine receptors (D2-DR). In addition we showed that in wild-type striatal neurons RGS9-2 and D2-DR had an identical cellular distribution pattern. These data support a role for RGS9-2 in suppressing the side effects associated with the treatment of psychoses and Parkinson's disease.

326. Number, density, and surface/cytoplasmic distribution of GABA transporters at presynaptic structures of knock-in mice carrying GAT1-GFP fusions

Chi-Sung Chiu, Kimmo Jensen[3], Irina Sokolova, Dan Wang[2], Ming Li, Purnima Deshpande, Norman Davidson, Istvan Mody[3], Michael W. Quick[2], Stephen R. Quake[1]

GAT1 molecules were counted near GABAergic synapses, to a resolution of ~0.5 μm. Fusions between GAT1 and green fluorescent protein (GFP) were tested in heterologous expression systems, and a construct was selected that shows function, expression level, and trafficking similar to that of WT GAT1. A strain of knock-in mice were constructed that express this mGAT1-GFP fusion in place of the WT GAT1 gene. The pattern of fluorescence in brain slices agreed with previous immunocytochemical observations. [^{3}H]GABA uptake, synaptic electrophysiology, and subcellular localization of the mGAT1-GFP construct were also compared with WT mice. Quantitative fluorescence microscopy was employed to measure the density of mGAT1-GFP at presynaptic structures in CNS preparations from the knock-in mice. Fluorescence measurements were calibrated with transparent beads and gels that have known GFP densities. Surface biotinylation defined the fraction of transporters on the surface versus those in the nearby cytoplasm. The data show that the presynaptic boutons of GABAergic interneurons in cerebellum and hippocampus have a membrane density of GAT1 800-1300 GAT1 molecules/mm^2, and the axons that connect boutons have a linear density of 640 GAT1 molecules/mm. A cerebellar basket cell bouton, a pinceau surrounding a Purkinje cell axon, and a cortical chandelier cell cartridge carry 9000, 7.8 million, and 430,000 GAT1 molecules, respectively. 61-63% of these molecules are on the surface membrane. In cultures from hippocampus, the set of fluorescent cells equals the set of GABAergic interneurons. Knock-in mice carrying GFP fusions of membrane proteins provide quantitative data required for understanding the details of synaptic transmission in living neurons.

[1]*Division of Engineering and Applied Physics, California Institute of Technology, Pasadena CA 91125*
[2]*Department of Biological Sciences, University of Southern California, Los Angeles CA 90089*
[3]*Departments of Neurology and Physiology, UCLA School of Medicine, Los Angeles, CA 90095*

327. GABA transporter-1-deficient mice: Differential tonic activation of $GABA_A$ versus $GABA_B$ receptors in the hippocampus

Kimmo Jensen[1], Chi-Sung Chiu, Istvan Mody[1]

Following its release from interneurons in cortex, the major inhibitory neurotransmitter GABA is taken up by GABA transporters (GATs). The predominant neuronal GABA transporter GAT1 is localized in GABAergic axons and nerve terminals, where it is thought to influence GABAergic synaptic transmission, but the details of this regulation are unclear. To address this issue, we have generated a strain of GAT1-deficient mice. We observed a large increase in a tonic postsynaptic hippocampal $GABA_A$ receptor-mediated conductance. There was little or change in the waveform or amplitude of spontaneous IPSCs or miniature IPSCs. In contrast, the frequency of quantal GABA release was one-third of WT, although the densities of $GABA_A$-receptors, $GABA_B$-receptors, GAD65 and VGAT1 were unaltered. The GAT1-deficient mice lacked a presynaptic $GABA_B$-receptor tone, present in WT mice, which reduces the frequency of spontaneous inhibitory postsynaptic currents. We conclude that GAT1 deficiency leads to enhanced extracellular GABA levels leading to an over activation of $GABA_A$-receptors responsible for a postsynaptic tonic conductance. Chronically elevated GABA levels also cause a downregulation of quantal GABA release and presynaptic signaling via $GABA_B$, thus causing a shift from quantal to tonic inhibition.

[1]*Department of Neurology, UCLA School of Medicine, Los Angeles, CA 90095*

328. GABA transporter (GAT1)-deficient mice display ataxia, tremor, reduced locomotor activity and an increased $GABA_A$ receptor-mediated tonic conductance in cerebellar granule cells

C.-S. Chiu, S. Brickley[1], K. Jensen[2], I. Mody[2]

We created GABA transporter subtype I-deficient mice (mGAT1 KO) by gene targeting. The mGAT1 KO mice reproduce and have normal muscle strength and life span, but reduced body weight (female -10%; male -20%), and motor disorders including gait abnormality, reduced time on rotarod, constant tremor at 25-32 Hz, and reduced locomotor activity inside home cage. In open field tests, mGAT1 KO mice display delayed exploratory activity, reduced rearing, and reduced visits to the central area, although without reduction in total distance traveled. These behaviors partially phenocopy effects of tiagabine, a GAT1-specific inhibitor, suggesting that they arise directly from GAT1 deficiency. The behavioral defects associated with excessive extracellular GABA in this animal illustrate the importance of GABA transporters in the regulation of correct neural function. Immunocytochemistry shows no detectable loss of GABAergic interneurons, no change in the number of $GABA_A$-, $GABA_B$-, or GAD65-immunoreactive structures and no change in GAT3 expression pattern in hippocampus and cerebellum, suggesting little or no compensatory expression of GABA transmission related proteins. This, the first animal carrying excess GABA in the central nervous system, serves a model for illustrating GABA function.

[1]*Pharmacology, University College London, London, United Kingdom*
[2]*Neurology, UCLA, Los Angeles, CA, USA*

329. Respiratory rhythm and pH sensitivity in Kir4.1 knock-out mice: Do inwardly rectifying potassium channels act as ph sensors?

C. Neusch[1], F. Kirchhoff[2], M. Bähr[1], S. Hülsmann[3]

Blood pH and CO_2 changes are compensated for by respiratory activity. This in turn is regulated by the firing frequency of inspiratory interneurons in the ventral respiratory group (VRG). The molecules involved in brainstem chemosensitivity are not yet identified.

pH/CO_2-sensitive Kir channels are likely candidates for chemosensors since they can directly detect pH/CO_2 changes and transmit the information to the membrane potential of the cell. Activation of Kir4.1 inward currents is known to become pH/CO_2 sensitive at physiological pH only when the ion pore is assembled from Kir4.1 and Kir5.1 subunits. Expression of the Kir4.1/5.1 channel heteromer has been demonstrated in respiratory brainstem nuclei by *in situ* hybridization. Here, we show that genetic inactivation of the Kir4.1 subunit in mice is followed by a dramatic change in postnatal breathing behavior. While breathing frequency increases in control mice from 3 Hz to 7 Hz over the first two postnatal weeks, the respiratory pattern in null mutants remains immature (breathing frequency of 3 Hz up to P12). Immunocytochemistry reveals Kir4.1 subunit expression in the VRG in control mice and vacuolation of respiratory brainstem areas in Kir4.1 null mutants. Our data suggest an important role of Kir4.1 channels in the development of respiratory pattern and the regulation of systemic pH/CO_2 changes by the respiratory network.

[1]*Department Neurol, Georg-August-University, Göttingen, Germany*
[2]*Department Neurogenetics, MPI for Exp Med., Göttingen, Germany*
[3]*Department Neuro and Sensory Physiology, Georg-August-University, Göttingen, Germany*

330. Open-state disulfide crosslinking between *M. tuberculosis* mechanosensitive channel subunits

George Shapovalov, Randal Bass[1], Douglas C Rees[1]

The mechanosensitive channel of large conductance from *M. tuberculosis* (Tb-MscL) was subjected to cysteine scanning mutagenesis at several residues in the M1 region. The V15C channel displayed crosslinking in air but not in the presence of 100 mM β-mercaptoethanol. In single-channel experiments, the V15C channel was more sensitive to tension than was wild-type MscL. In air, Tb-MscL V15C occasionally displayed "signature events," at constant tension, there was first a sojourn in the highest-conductance open state, then a series of transitions to substates. During a signature event, these transitions do not appear to be reversible. Some sojourns in the lower-conductance states lasted for $\geq$ 100 sec. These signature events were abolished by 100 mM β-mercaptoethanol and did not occur in a cysteineless gain of function mutant, suggesting that the signature events represent disulfide crosslinking between channel subunits. We conclude that the crosslinking occurs during an open state, during asymmetric sojourns that bring the α-carbons of adjacent 15C side chains within 3.6 to 6.8 Å. Such asymmetric structures must be considered in models of TB-MscL gating.

[1]*Division of Chemistry and Chemical Engineering*

Publications

Beene, D.L., Brandt, G.S., Zhong, W., Zacharias, N.M., Lester, H.A. and Dougherty, D.A. (2002) Cation-interactions in ligand recognition at serotonergic ($5\text{-}HT_{3A}$) and nicotinic acetylcholine receptors. The anomalous binding properties of nicotine. *Biochemistry* **41**:10262-10269.

Chiu, C.-S., Jensen, K., Sokolova, I., Wang, D., Li, M., Deshpande, P., Davidson, N., Mody, I., Quick, M.W., Quake, S.R. and Lester, H.A. (2002) Counting GABA transporters at presynaptic terminals of knock-in mice carrying GAT1-GFP fusions, and tremors in GAT1 knockout mice. Accepted for publication with modifications, *J. Neurosci*. In press.

Eickhorst, A., Berson, A., Cockayne, D., Lester, H.A. and Khakh, B.S. (2002) Control of P2X2 channel permeability by the cytosolic domain. *J. Gen. Physiol*. **120**:119-131.

Jensen, K., Chiu, C-S., Lester, H.A. and Mody, I. GABA transporter-1 deficient mice: Differential tonic activation of $GABA_A$ versus $GABA_B$ receptors in the hippocampus. Submitted for publication.

Kovoor, A. and Lester, H.A. (2001) Gi Irks GIRKs. *Neuron* **33**:6-8.

Li, M. and Lester, H.A. (2002) Early fluorescence signals detect transitions at mammalian serotonin transporters. *Biophys. J.* **83**:206-218.

Li, M., Farley, R.A. and Lester, H.A. (2002) Voltage-dependent transient currents of human and rat 5-HT transporters (SERT) are blocked by HEPES and ion channel ligands. *FEBS Lett*. **513**:247-252.

Li, P., Slimko, E.M. and Lester, H.A. Selective elimination of glutamate activation and introduction of fluorescent proteins into a *C. elegans* chloride channel. *FEBS Lett*. In press.

Nadeau, H. and Lester, H.A. (2002) NRSF causes cAMP-sensitive suppression of sodium current in cultured hippocampal neurons. *J. Neurophysiol*. **88**:409-421.

Neusch, C., Rozengurt, N., Kofuji, P. and Lester, H.A. Inner ear defects and deafness in mice lacking the Kir4.1 potassium channel subunit. Submitted for publication.

Owens, J.C., Balogh, S.A., McClure-Begley, T.D, Labarca, C., Lester, H.A., Picciotto, M.R., Wehner, J.M. and Collins, A.C. α4β2 neuronal acetylcholine receptors: Targets for both nicotine and ethanol. Submitted for publication.

Petersson, E.J., Brandt, G.S., Zacharias, N.M., Dougherty, D.A. and Lester, H.A. (2002) Caging proteins through unnatural amino acid mutagenesis. *Meth. Enzymol. Biophotonics*. In press.

Petersson, E.J., Choi, A., Dahan, D.S., Lester, H.A. and Dougherty, D.A. A perturbed pK_a at the binding site of the nicotinic acetylcholine receptor: Implications for nicotine binding. Submitted for publication.

Petersson, E.J., Shahgholi, M., Lester, H.A. and Dougherty, D.A. (2002) MALDI-TOF mass spectrometry methods for evaluation of *in vitro* aminoacyl tRNA production. *RNA* **8**:542-547.

Slimko, E.M., McKinney, S., Anderson, D.J., Davidson, N. and Lester, H.A. (2002) Selective electrical silencing of mammalian neurons *in vitro* using invertebrate ligand-gated chloride channels. *J. Neurosci*. In press.

Professor of Biology: Paul H. Patterson
Research Fellows: Romulo de Castro, Kristina Holmberg, Bradley Kerr, Ali Khoshnan
Graduate Student: Jennifer Montgomery
Research and Laboratory Staff: Jing Han, Garrett Heffner[1], Jan Ko, Doreen McDowell, Floreen Rooks, Limin Shi, Nora Tu[1]
[1]*Undergraduate student, Caltech*

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Summary: Much of our research involves the study of neuroimmune interactions. Using knockout (KO) mice and over-expression *in vivo* with viral vectors, we are exploring the role of neuropoietic cytokines in synaptic activity and plasticity, PNS and CNS injury and repair, epilepsy, Alzheimer's disease, pain and inflammation. Another aspect of neuroimmune regulation of pathology is being investigated in our experiments of the mechanism of how stress affects melanoma tumor progression. In a study 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. Additional projects center on Huntington's disease (HD), and the use of recombinant single chain antibodies to perturb huntingtin function and toxicity.

Cytokines are diffusible, intercellular messengers that were originally studied in the immune system. Our group has contributed to the discovery of a new family that we have termed the neuropoietic cytokines, because of their action in both the nervous and hematopoietic/immune systems. We have demonstrated that one of these cytokines, leukemia inhibitory factor (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, both within and outside the nervous system. We are exploring the implications of these findings in the study of animal models of nerve and spinal cord injury and regeneration, epilepsy and inflammatory pain. We find, for instance, that LIF is a critical regulator of astrocyte activation following stroke or seizure, and that this cytokine also regulates inflammatory cell infiltration, neuronal death, gene expression, and the production of new neurons from stem cells following injury. These results highlight LIF as an important therapeutic target. We are also examining the role of related cytokines in a transgenic mouse model of Alzheimer's disease.

Cytokines may also be involved in the modulation of connections during the synaptic plasticity that underlies learning and memory. We have examined the role of LIF and related cytokines in hippocampal long-term potentiation (LTP) in slices and *in vivo*. Results in the latter preparation suggest an involvement of immune cells in LTP. This work involves KO mice, electrophysiology, and delivery of recombinant proteins or the genes that code for them.

Cytokines involvement in a new model for mental illness is also being investigated. This mouse model is based on epidemiological findings that maternal viral infection can increase the likelihood of schizophrenia or autism in the offspring. We are using behavioral, molecular, neuropathological and brain imaging methods to investigate the effects of maternal influenza infection on fetal brain development and altered behavior in adult offspring.

We are utilizing intracellular antibody expression to block the toxicity of mutant huntingtin, the protein that causes HD. We have produced single-chain antibodies (scFvs) that bind to various domains of huntingtin, and these can either exacerbate or alleviate huntingtin toxicity in cultured cells. Work has begun on delivering these scFvs using viral vectors in a mouse model of HD.

331. Regulation of adult neural stem cell proliferation and differentiation by leukemia inhibitory factor (LIF) *in vivo*

Bradley J. Kerr, Jing Han, Paul H. Patterson

There are neural stem cells and restricted progenitors residing in particular areas of the adult mammalian brain. It would be of considerable therapeutic interest to be able to control the proliferation and differentiation of these cells in response to injury or disease. Previous studies from our own and other laboratories have shown that LIF can regulate the proliferation of neural stem cells *in vitro*, and stimulate neurogenesis in the olfactory epithelium *in vivo*. We now report that, compared to LacZ and PBS controls, intraventricular injection of a recombinant adenoviral construct expressing LIF significantly enhances bromodeoxyuridine labeling in and around the ependyma lining the lateral, third and fourth ventricles. Enhanced labeling is also seen in another neurogenic zones, the dentate gyrus of the hippocampus, as well as in the olfactory bulb, striatum and cortex. Thus, LIF may be able to regulate progenitor cell proliferation and fates in the CNS *in vivo* as it does *in vitro*.

332. The role of LIF in regulating the inflammatory cascade following spinal cord injury

Bradley J. Kerr

Leukemia inhibitory factor (LIF) is a critical mediator of the inflammatory response in the nervous system and in peripheral tissue. We are exploring how LIF regulates the inflammatory cascade after spinal cord injury, with over-expression of LIF using a recombinant adenoviral construct, and also LIF knockout mice. Reactive gliosis, inflammatory cell infiltration and the expression of pro-inflammatory cytokines and chemokines are being assayed. These studies are also aimed at determining how the inflammatory cascade affects the degree of secondary tissue injury and the regenerative capacity of axons after spinal cord damage.

333. LIF is required for astrocyte activation and neuropeptide responses to seizure

Kristina H. Holmberg

Our group previously found that the cytokine leukemia inhibitory factor (LIF) is induced in astrocytes in response to seizure (Jankowsky and Patterson, 1999). We are now using adult LIF knockout (KO) mice to study the function of LIF in post-seizure sequelae in the hippocampus. Twenty-four hours after seizure, immunocytochemical staining (ICC) for glial fibrillary acidic protein (GFAP) is up-regulated in the wild type (WT), but not in the KO. This indicates that LIF is autocrine regulator of astrocyte activation following seizure, as it is following physical injury to the cortex (Sugiura *et al*., 2000). LIF also appears to be important for the changes in expression of several neuropeptides that occur in response to seizure. While seizure reduces calretinin ICC in the supragranular layer in WT mice, we see little change in the LIF KO. In contrast, staining for neuropeptide Y in area CA3 is much higher in the LIF KO than in WT following seizure. Moreover, the number of NPY-positive neurons in certain cortical areas is higher in the LIF KO following seizure than in WT. This evidence indicates that LIF is critical for changes in neuropeptide expression caused by seizure, as it is for neuropeptide responses to axotomy in peripheral neurons.

334. New anti-huntingtin monoclonal antibodies: Implications for huntingtin conformation and its binding proteins

Jan Ko, Susan Ou, Paul H. Patterson

Many neurodegenerative diseases are characterized by misfolded, aggregation-prone proteins. Polyglutamine (polyQ) diseases, including Huntington's disease (HD), are caused by expansion of the trinucleotide repeats coding for glutamate. A common feature of the polyQ diseases is the formation of intranuclear inclusions or aggregates, which are found in brains of both animal models and patients. We produced a series of new monoclonal antibodies (mAbs) that bind to various regions of the mutant protein that causes HD, huntingtin (Htt). Peptide array epitope mapping shows that mAbs MW1-6 specifically bind to the polyQ domain of Htt exon 1. On Western blots MW1-5 all strongly prefer to bind to the expanded polyQ repeat form of Htt. Despite sharing the same epitope and binding preferences on Western blots, MW1–5 display distinct staining patterns in immunohistochemistry with wild-type (WT) and mutant Htt transgenic mouse (R6) brains. This suggests that in its various subcellular locations, the polyQ domain of Htt either takes on different conformations and/or is differentially occluded by Htt binding proteins. MAbs MW7 and 8 can differentiate transgenic from WT mice by staining nuclear inclusions in R6/2 brain. Epitope mapping reveals that MW7 and 8 specifically bind the polyP domains and amino acids 83-90 of Htt, respectively. In collaboration with Ron Wetzel (Univ. Tenn.), we have also produced a new series of anti-Htt mAbs that bind preferentially to polyQ domains with different structures. Some of these mAbs display staining patterns in the brain that are distinct from the MW mAb series.

335. Mutant huntingtin activates NF-κB through interaction with IKKγ

Ali Khoshnan, Jan Ko

Transcriptional dysregulation and enhanced susceptibility to excitotoxicity of striatal neurons have been implicated in the pathogenesis of Huntington's disease (HD). It is not known if these two mechanisms are related, however. We find that the NF-κB transcriptional pathway is activated by mutant huntingtin protein (Htt) in PC12 cells. Activation is mediated by physical interaction between mutant Htt exon-1 and IKKγ, a regulatory component of the IκB kinase complex (IKK). This interaction results in activation of the IKK complex, involving phosphorylation and degradation of the inhibitory protein IκB. A recombinant antibody specific for the polyproline domain of Htt interferes with binding of mutant Htt to the IKK complex and diminishes Htt-induced NF-κB activation, suggesting that this domain is responsible for the association. Moreover, a Htt construct lacking the polyP domain fails to bind to IKKγ. Association of these molecules is also indicated in the brain, as we find that IKKγ co-localizes with Htt in neurons of HD transgenic mice. Since ligand binding to the NMDA receptor is known to activate the NF-κB pathway in striatal neurons, we propose that mutant Htt, through its interaction with IKKγ, participates in the selective vulnerability of striatal neurons to excitotoxicity in HD.

336. The effect of restraint stress on the immune system and melanoma progression

Jennifer Montgomery, Paul H. Patterson

We are investigating the relationship between stress, the immune system and melanoma tumor progression. Using a murine model of restraint stress, we are initially monitoring changes in immune function in normal animals as well as in a new genetic model of

melanoma that is based on the genetics of human tumors. In this model, cutaneous melanoma occurs spontaneously at a predictable time, allowing the study of the disease *in situ* as it naturally progresses. We are currently assaying changes in serum cytokine levels caused by stress. Specifically, we are looking at IL-1β, TNF-α and IL-6, as they have been implicated in both the stress response as well as in melanoma growth in culture. Other parameters being quantified include corticosterone, which is released in response to stress, and natural killer cells, which are important in tumor surveillance.

337. Developing a viral model for schizophrenia and/or autism

Limin Shi, Paul H. Patterson

Exposure of pregnant women to viral infection during a critical period can increase the incidence of schizophrenia and possibly autism in their offspring. We previously reported that influenza respiratory infection of pregnant mice causes highly abnormal behavioural responses in their offspring, tested as adults. To investigate neuropathological correlates of these behaviours, and to compare with the human pathology of these disorders, we are studying the histology of the brains of adult mice born to infected mothers. These offspring display a loss of Purkinje cells specifically in lobule VII and of the cerebellar vermis. We also occasionally see what appear to be misplaced Purkinje cells in the white matter of this lobule. In addition, the molecular layer of lobule VII appears to be thinner where the Purkinje cells are missing. This neuropathology is of particular interest because it is very similar to that reported for autistic brains, both in imaging and post-mortem studies. It is also possible that this finding in the mouse model is relevant to the pathology and behavioural abnormalities that have been linked to the cerebellum in schizophrenia. We are also labelling mice with ^{14}C-fluorodeoxyglucose (FDG), which is a marker of cerebral glucose metabolism. FDG uptake can reveal alterations in the activity or density of synaptic terminals. In the mice born to infected mothers, we are looking for hyper-function (as in the positive symptoms of schizophrenia – delusions and hallucinations), or lower function (as in the negative symptoms of schizophrenia and autism) in various brain areas.

Publications

Bauer, S., Han, J., Rasika, S., Mauduit, C., Jourdan, F., Benahmed, M., Moyse, E. and Patterson, P.H. (2002) Leukemia inhibitory factor induction is a key signal of lesion-triggered neurogenesis in the adult mouse olfactory epithelium. *J. Neurosci*. In press.

Fatemi, S.H., Earle, J., Kanodia, R., Kist, D., Emamian, E.S., Patterson, P.H., Shi, L. and Sidwell, R. (2002) Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: Implications for genesis of autism and schizophrenia. *Cell Mol. Neurobiol*. **22**:25-33.

Khoshnan, A., Ko, J. and Patterson, P.H. (2002) Effects of intracellular expression of anti-huntingtin antibodies of various specificities on mutant huntingtin aggregation and toxicity. *Proc. Natl. Acad. Sci. USA* **99**:1002-1007.

Ko, J., Ou, S. and Patterson, P.H. (2001) New anti-huntingtin monoclonal antibodies: Implications for huntingtin conformation and its binding proteins. *Brain Res. Bull*. **56**:319-329.

Patterson, P.H. (2002) Maternal infection: Window on neuroimmune interactions in fetal brain development and mental illness. *Curr. Opin. Neurobiol*. **12**:115-118.

Shi, L., Fatemi, S.H., Sidwell, R.W. and Patterson, P.H. (2002) A mouse model of mental illness: Maternal influenza infection causes behavioral and pharmacological abnormalities in the offspring. *J. Neurosci.*, in press.

Tofaris, G.K., Patterson, P.H., Jessen, K.R. and Mirsky, R. (2002) Denervated Schwann cells attract macrophages by secretion of LIF and MCP–1 in a process regulated by IL-6 and LIF. *J. Neurosci*. **22**:6696-6703.

Associate Professor: Erin M. Schuman
Visiting Associate: Adam Mamelak[1]
Visiting Professor: Karen D. Parfitt[2]
Postdoctoral Fellows: Changan Jiang, Hyong Kyu Kim, Sachiko Murase, Gentry Patrick, Shaojun Tang
Graduate Students: Girish Aakalu, Baris Bingol, Jessica Edwards, Vivek Jayamaran, Eric Mosser, Armando Miguel Remondes[3], W. Bryan Smith
Technical Staff: Chiranjib Dasgupta, Michael Goard, Chris Park, Holli Weld
Administrative Staff: Ana Maria Lust, Alana Rathbun
Undergraduate Students: Audrey Carstensen[4], Nalini Colaco[4], Melinda Owens[4], Nick Wall[4]
[1]*Huntington Memorial Hospital, City of Hope*
[2]*Pomona College, Department of Biology*
[3]*Graduate student, Instituto Gulbenkian de Ciência, Oeiras, Portugal*
[4]*Undergraduate students, Division of Biology, California Institute of Technology*

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Summary: Synapses, the points of contact and communication between neurons, can vary in their size, strength and number. The differences in synapses and their ability to change throughout the lifetime of the animal contribute to our ability to learn and remember. We are interested in how synapses are modified at the cellular and molecular level. We are also interested in how neuronal circuits change when synapses change their properties. We conduct all of our studies in the hippocampus, a structure known to be important for memory in both humans and animals. We use molecular biology, electrophysiology and imaging to address the questions detailed below.

A major focus of the lab concerns the cell biological mechanisms that govern modifications at individual synaptic sites. In particular, we are interested in the idea that dendritic protein synthesis and degradation may contribute to synaptic plasticity. We are also interested in mRNA and protein trafficking during synaptic plasticity.

We are also examining the role of the cadherins family of cell adhesion molecules in synaptic plasticity. Several labs have shown that cadherins are localized to synapses in the hippocampus. Earlier, we demonstrated that function-blocking cadherin antibodies or peptides can prevent long-term potentiation, without interfering with basal synaptic transmission. We hypothesize that cadherin bonds may be sensitive to local fluxes in extracellular calcium imposed by action potential activity. We are now examining the molecular mechanisms by which cadherins influence synaptic strength and the involvement of cadherins in the formation and maintenance of synapses, using fluorescence resonance energy transfer. We have recently shown that β-catenin, the intracellular signaling partner for cadherin, undergoes a dynamic redistribution upon depolarization. β-catenin moves from spines to shafts; its movement is controlled by the phosphorylation state of a tyrosine residue. Furthermore, β-catenin orchestrates a local reorganization of synaptic area in spines, as well as associated pre-synaptic terminals.

We are interested in how information is processed in the brain. In particular, how does the circuitry of the hippocampus gate or influence information flow? Information reaches the CA1 region of the hippocampus via the conventional trisynaptic loop as well as via a direct input from entorhinal cortex. We are examining how these two inputs interact and influence the output of CA1 pyramidal neurons. Lastly, we are interested in human hippocampal function. In particular, we are conducting multi-unit recordings from human hippocampus *in vivo* during learning and memory tasks to determine whether changes in spike activity are correlated with behavior.

338. Regulation of local protein synthesis by neural activity

Chiranjib Dasgupta, Changan Jiang

Local protein synthesis plays critical roles in the induction of long-lasting forms of synaptic plasticity, but the mechanisms by which neural activity triggers translation in dendrites are largely unclear. Recent studies have suggested that the translatability of some dendritically localized mRNAs, such as CaMKIIα mRNA, is controlled by cytoplasmic polyadenylation. A key player in this process is Cytoplamic Polyadenylation Element-Binding Protein (CPEB). Normally, CPEB binds the CPE-containing mRNAs and represses their translation. Phosphorylation of CPEB in response to synaptic activity converts it into a translational activator, which subsequently induces poly(A) elongation and translation initiation of the bound mRNAs. To further study the regulation of poly(A) length by synaptic activity, we are examining the functions of two other proteins, Pumilio and Brunol, in the translation of dendritic mRNAs. Both proteins function as translational repressors in *Drosophila* and *Xenopus*, possibly through the shortening of poly(A) tails of the target mRNAs. Our preliminary results indicate that Pumilio and Brunol are present in the dendrites of hippocampal neurons. Their localization patterns only partially overlap, suggesting that they may regulate the translation of distinct sets of dendritic mRNAs. Currently, we are examining whether these two translational repressors exert their functions through interacting with CPEB and whether their activities are regulated by synaptic activity.

339. Studying dendritic protein synthesis in hippocampal slices and living animals

Changan Jiang

Our recent study using GFP-based reporters has provided strong evidence that synaptic activity can induce protein synthesis in the dendrites of cultured hippocampal neurons. To extend our study into a more physiological context, such as in hippocampal slices and in living animals, we are generating transgenic mice to express protein synthesis reporters in a tissue- and temporal-specific manner. In the first lines of transgenic animals, we use CaMKIIα promoter to drive the expression of rtTA, a tetracycline-dependent transcription activator, in hippocampal neurons. In the other two lines of transgenic animals, we fuse GFP with either actin (GFP-ACT) or a canonical myristoylation signal (myrGFP) and put the fusion genes under the control of a tetracycline-sensitive promoter. By breeding the rtTA animals with the GFP-ACT or myrGFP animals, we have obtained double-transgenic animals with both rtTA and GFP reporter transgenes. They allow us to transiently induce the expression of GFP reporters in their hippocampi by feeding them with doxycycline. Currently, we are evaluating whether these transgenic animals are suitable for our studies of local protein synthesis during long-term potentiation in hippocampal slices and long-term memory formation in living animals.

340. Role of dendritic protein synthesis in hippocampal LTP

Melinda Owens

Local protein synthesis in the dendrites provides a logical mechanism for achieving and maintaining specificity during synaptic enhancement. It has been shown that the neurotrophic factor BDNF can induce protein synthesis in physically and "optically" isolated dendrites (Aakalu *et al*., 2001). However, as of yet, the connection between dendritic protein synthesis and specific patterns of neural activity has not been firmly established. Using a diffusion limited GFP-based dendritic protein synthesis reporter that contains the 5' and 3' UTRs of CaMKIIα, we are characterizing the relationship between dendritic protein synthesis and synaptic activity in the hippocampus. Hippocampal neurons, first in culture and then in slices, will be infected with the reporter and stimulated with different electrical patterns associated with the induction of synaptic plasticity. Then, changes in the expression level and distribution of the GFP reporter will be imaged and analyzed. Several different stimulation protocols will used because different protocols for inducing plasticity use different molecular mechanisms (Kang *et al*., 1997) and might differentially stimulate dendritic protein synthesis as well. Some protocols we will try include stimulation at 100 Hz, theta burst stimulation, and pairing depolarization and low frequency stimulation. The duration of the stimulation will also be varied to further elucidate the relationship between synaptic plasticity and protein synthesis in the dendrites.

341. The effects of NMDA and dopamine receptor agonists on local protein synthesis and input-specific synaptic enhancement

W. Bryan Smith, Nicholas R. Wall, Karen Parfitt

It is well established that *de novo* protein synthesis is required for long-term memory storage. Furthermore, the activity-dependent regulation of local protein synthesis (LPS) in the dendritic compartment is an attractive candidate mechanism for regulating the persistence and spatial specificity of late-phase long-term potentiation (LTP). Recent work in the lab has demonstrated that isolated dendrites of mature hippocampal neurons are able to synthesize proteins (Aakalu and Smith *et al*., 2001), but the role of synaptic activity in driving input-specific protein synthesis remains to be determined.

Our preliminary data indicate that bath application of NMDA or the D1/D5 agonist SKF-38393 is capable of increasing GFP synthesis in cultured hippocampal neurons. In order to demonstrate a causal relationship between protein synthesis induction and the input specificity of synaptic enhancement, we are examining the effects of targeted delivery of these compounds on LPS induction. Using a chemically caged variant of NMDA and a fiber optic setup for precise delivery of the UV uncaging light, we can activate NMDA receptors with a spatial precision of 5-10 μm. This technique will allow us to explore more precisely the relationship between local synaptic activity and dendritic protein synthesis activation. In addition to the glutamate-mediated synaptic activity, we are interested in the possible modulatory effects of dopamine receptor activation on LPS. By combining local NMDA uncaging with dopamine agonist (SKF) micro perfusion we will begin to understand how these receptor populations regulate the activity-driven dynamics of dendritic protein synthesis.

Our studies on focal activation of LPS are hampered by the use of GFP due to the time required for maturation of the biological fluorophore, which may require up to 30 minutes. If relatively small quantities of GFP are synthesized in response to synaptic activation we may not be able to detect the increases locally as the GFP may diffuse away from the area of interest before becoming fluorescent. We are currently investigating the utility of various protein synthesis reporters based on puromycin, an amino-acyl tRNA analog (collaboration with Rich Roberts and Shelley Starck, Division of Chemistry). Bath application of a fluorescein-puromycin compound to SKF-treated neurons results in a significant increase in fluorescein incorporation when compared with untreated control neurons. Although further characterization is required, the use of puromycin-based protein synthesis reporters may prove useful in dissecting the spatial and temporal characteristics of activity-induced LPS.

342. Synaptic silencing results in increased dendritic protein synthesis

Girish Aakalu, Michael Goard

Our lab has previously found that stimulating cultured neurons with BDNF causes an increase in local protein synthesis, shown using a diffusion limited GFP-based protein synthesis reporter (Aakalu *et al*., 2001). In order to investigate the effect of intrinsic synaptic activity on our reporter, we treated infected neurons with a cocktail of pharmacological agents that block synaptic activity, expecting reporter expression to decrease. However, we found that the expression of the reporter increased in distal dendrites after blocking synaptic activity for 48 hours. This finding, though counterintuitive at first glance, is actually in line with previous reports that synaptic activity blockade results in a long-lasting form of compensatory synaptic plasticity known as "disuse hypersensitivity" or "homeostatic plasticity." Preliminary experiments have suggested that the increase in reporter expression is apparent in the first few hours after application of synaptic activity blockers, though it takes much longer for the compensatory plasticity to manifest itself. We will use time lapse imaging to elucidate the role of the increased dendritic protein synthesis in homeostatic plasticity. We hope to determine whether this acute increase in dendritic protein synthesis is necessary for the homeostatic plasticity, as well as elucidating the molecular mechanisms leading to the increase (e.g., whether the increase in protein synthesis is repressed by native activity or triggered by some sort of biological sensor of low levels of synaptic activity). These experiments should provide insight into the cellular mechanisms of native synaptic plasticity and regulation.

343. Role of pumilio in dendritic protein synthesis

Chiranjib Dasgupta

The PUF-family of proteins has been implicated as modulators of several developmental programs; these modulatory effects are exerted by repression of translation. This group of proteins are ubiquitously expressed and highly conserved in all species starting from yeast to human. The feature that is special and common to all PUF proteins is the presence of a unique RNA binding domain (RBD) that recognizes specific sequences on the 3'-untranslated region (3'-UTR) of developmentally critical mRNAs for translational repression. The RBD is also called PUF motif. The two lead members of this protein family Pumilio and FBF have been studied extensively in *Drosophila melanogaster* (Dm) and *Caenorhabditis elegans*. In fruitfly, during differentiation, pumilio along with nanos and brain-tumor represses translation of the *hunchback* (*hb*) mRNA by binding to a unique bipartite motif at the 3'-UTR known as the nanos response element (NRE). Besides the *hb* mRNA, *cyclin B* mRNA is also a target of pumilio in the fruitfly. Recent studies on *Xenopus levis* have indicated *cyclin B1* mRNA to be a target of pumilio also. Interestingly, the *cyclin B1* mRNA does not contain any bipartite NRE, but contains instead a single UGUA tetranucleotide sequence in the 3'-UTR that was shown to be required for pumilio binding. We are investigating if pumilio might play a regulatory role in dendritic protein synthesis.

Our studies revealed that pumilio is present in the rat hippocampus; we have cloned the entire RBD and raised antisera against this protein. Using this antisera we showed pumilio is expressed in soma and dendrites in cultured neurons. This is consistent with the recent reports of pumilio distribution in *Drosophila* neurons. Since the PUF domain of Dm-pumilio is sufficient for translational repression, we first expressed the EGFP-rPUF fusion protein in cultured neurons and observed the fusion protein to be expressed in soma as well as in dendrites. In addition, we created EGFP-full-length pumilio fusion protein and two different *pdsred2*-based reporters wherein the 3'UTR of *pdsred2* (Clontech) was modified to contain either a 150 base pair (bp) *hb* cDNA 3'-UTR derived sequence that harbors the intact bipartite NRE or the entire 110 bp *X. levis cyclin B1* 3'-UTR that harbors two cytoplasmic polyadenylation elements (CPEs), CPE1 and CPE2. The CPE sequences are the binding sites for CPEB. With the help of these fluorescently-tagged fusion proteins, we are currently developing a co-transfection assay in neurons to evaluate if pumilio may repress the translation of these reporters. Eventually, we will express the RNA binding domain mutants of pumilio in neurons using this assay system to examine if they exert a dominant negative effect. Furthermore, since the 3'-UTR of the dendritically important CamKIIα-mRNA, like *cyclin B1*, contain UGUA motif and the CPEs, we would like to identify other dendritically localized mRNAs whose translation is regulated by pumilio and CPEB. Eventually, we shall express full-length pumilio, pumilio-RBD and their respective dominant-negative mutated forms in organotypic slices and in whole animals to examine their effects on dendritic protein synthesis, synaptic plasticity and animal behavior.

344. Methods for quantitative analysis of colocalization and spatial correlation in multi-channel immunofluorescence images

*W. Bryan Smith, Vivek Jayaraman, Gilles Laurent**

Fluorescence microscopy has become an essential tool for the study of protein distributions in neurons. With the use of multi-channel immunofluorescence microscopy, it is possible to investigate the distributions of two or three distinct populations of proteins relative to one another. Frequently the fluorescence signals are colocalized, implying a functional relationship between the proteins under investigation. Although protein colocalization is often reported, the amount of colocalization is rarely quantified. To address this issue, we have developed a series of simple, automated MATLAB routines that perform quantitative measures of 2-D and 3-D colocalization on multi-channel confocal fluorescence micrographs.

In addition to colocalization, clusters of proteins may also exhibit some degree of spatial correlation. Given

the rapid diffusion and active translocation of many cellular proteins, this spatial correlation is likely to be significant. For example, ribosomes that tend to cluster near the base of dendritic spines show little or no colocalization with synaptic markers, yet their consistent presence in close proximity to synapses is biologically relevant. In order to quantitate the correlation between such spatially related signals, we have devised a method for normalization of the 2-D cross-correlation function. For a given image pair, the punctate fluorescent signals are randomly redistributed 32 times within the image space. The arithmetic mean of the resulting 1024 randomized 2-D correlation matrices is then subtracted from the 2-D cross-correlation matrix of the original images. The resulting correlation matrix is a robust estimate of the spatial correlation of the two signals that does not depend on the amount of each signal in the original images. Using these image analysis routines, we are able to quantitatively investigate the dynamics of protein distributions in large populations of cultured neurons.

*Professor of Biology and Computation and Neural Systems, Caltech

345. Function of staufen in dendritic mRNA transport

Hyong Kyu Kim, Nalini Colaco

In neurons, the dendritic transport and subsequent translation of certain mRNAs play important roles in synaptic plasticity. Recent evidence suggests that mRNAs are transported by forming RNA granules, an aggregate of mRNA, ribosomal proteins, rRNA, and delivered to dendritic region by an RNA-binding protein, called staufen. To study the functions of staufen in dendritic transport of RNA granules, we are using a dominant-negative form of the staufen protein (stau-RBD) that contains the RNA-binding domains, but lacks the microtubule-association domain. We have found that overexpression of stau-RBD significantly decreases the levels of ribosomes in dendrites, but increases those in the soma, as revealed by Y10B immunostaining. This result suggests that truncated staufen binds the RNA granules and traps them in the cell body. We are currently repeating these experiments using additional ribosome-specific markers such as ribosomal L4 and S6 proteins. We have also found that the transport of RNA granules to the dendrites increases in response to neuronal activity. Depolarization of neurons by a high concentration of KCl or inhibiting GABA receptors increases Y10B signal intensity in the distal dendrites. To determine whether staufen is required for this increase, we are going to overexpress the wild-type or the dominant-negative forms of staufen in neurons and examine their effect on the change of RNA granule transport in response to external stimuli. We expect results from these studies will help us understand the regulation of mRNA transport to dendrites.

346. Isolation and characterization of locally synthesized proteins in neuronal dendrites

Hyong Kyu Kim, Shelley R. Starck, Rich Roberts***

Local protein synthesis in neuronal dendrites has been proposed as one mechanism by which neuronal activities induces long-lasting changes in synaptic efficacy. Although it is clear that local protein synthesis is required for at least some forms of LTD and LTP, the identities of the proteins synthesized in the dendrites remain largely unknown. To solve this problem, we are developing a high-throughput screening method to isolate locally synthesized proteins. First, biotin-conjugated puromycin (Biotin-2P), an aminoacyl-tRNA analog that can be incorporated into the specific position of newly synthesized peptides, is added to cultured neurons to tag newly synthesized proteins. Next, dendrites are isolated and the tagged proteins are purified by biotin-avidin affinity chromatography. Finally, MADI-TOF mass spectrometry will be used to characterize the proteins. Our preliminary result showed that we can use Biotin-2P to isolate newly synthesized GFP from cultured hippocampal neurons. Currently, we are optimizing the tagging and purification procedures. We expect that the results obtained from this study will further our understanding of synaptic plasticity at the molecular level.

*Chemistry grad student, Caltech
**Assistant Professor of Chemistry, Caltech

347. A method to locally control protein synthesis

Girish Aakalu, Olesya Fedoryak[1], Michael Goard, Carlo Quinonez, Jamii St. Julien[2], Stephen J. Poteet[1], Tim Dore[3]

The targeting of mRNA and localized synthesis of proteins to specific cellular domains enables cells to carry out asymmetric functions. In neurons, for example, the local synthesis of proteins may allow specific regions of dendrites to show long-lasting protein synthesis-dependent plasticity, such as long-term potentiation. In the case of LTP, the relative contributions of somatic vs. dendritic protein synthesis have been difficult to address because existing techniques do not permit selective inhibition in either cellular compartment. In order to inhibit protein synthesis in specific cellular domains (e.g., dendrites vs. cell bodies), we have developed a photo-releasable anisomycin compound. The cage consists of a 6-bromo-7-hydroxycoumarin-4-ylmethyl group, which can be removed through exposure to UV light. Therefore, the area of protein synthesis inhibition can be restricted to a small region defined by the limits of UV light exposure, or, potentially, multiphoton excitation if a pulsed IR laser is the light source. We have tested the compound's effectiveness using *in vitro* protein translation systems and cultured CHO cells. These tests indicate that the uncaged compound can effectively inhibit protein synthesis in a spatially restricted manner and does not affect protein synthesis in its caged form. This ability to "locally" inhibit protein synthesis will enable experiments in brain slices and cultured cells where one can specifically inhibit

protein synthesis in the cell body or synaptic layers. These experiments will elucidate the roles of somatic and/or dendritic protein synthesis during plasticity with both spatial and temporal precision.

[1]*Chemistry graduate student, University of Georgia*
[2]*SURF student, 2001*
[3]*Professor, University of Georgia, Chemistry Department*

348. Ubiquitin-mediated proteasome activity in glutamate receptor trafficking

Gentry N. Patrick, Baris Bingol, Holli A. Weld, Miguel Remondes

Although much attention has been paid to the role of protein synthesis in synaptic plasticity, relatively little is known about the potential role of protein degradation. The ubiquitin-conjugation system serves to identify, modify and deliver proteins to the proteasome for degradation. Using fluorescence immunocytochemistry, we found that both the proteasome and ubiquitin are present in the soma and dendrites and occasionally observed in dendritic spines of cultured hippocampal neurons. The presence of these proteins near synapses prompted us to ask whether ubiquitin-mediated protein degradation plays a role in synaptic plasticity. The internalization of glutamate receptors (GluRs) has been proposed to underlie some forms of synaptic plasticity, including long-term depression (LTD). Recent studies in other cell types have shown that proteasome activity can regulate the agonist-induced internalization of receptors. Thus, we examined whether blocking proteasome activity changes the pattern of agonist-induced GluR internalization in cultured hippocampal neurons. In agreement with published work, short-term treatment with the glutamate agonist AMPA induced a robust internalization of GluRs, detected immunohistochemically.

In contrast, brief pretreatment with a proteasome inhibitor dramatically reduced the amount of GluR internalization induced by AMPA. Inhibition of the proteasome did not affect the GluR surface receptor population prior to AMPA treatment. In agreement with block of GluR internalization observed during proteasome inhibition, the expression of a chain elongation-defective ubiquitin mutant (Ub-K48R) in hippocampal neurons severely diminished AMPA-induced internalization. Surprisingly, there was no effect of proteasome inhibitors on LTD induced in hippocampal slices. Taken together, these data indicate a role for regulated proteolysis in activity-dependent AMPA receptor dynamics but suggest that the internalization of GluRs may not be required for synaptic depression.

349. Visualization of cadherin-cadherin association in neurons

Eric Mosser

Classic cadherins, in particular N- and E-cadherins, are expressed and localized at synaptic sites of the adult rat hippocampus and involved in LTP. Cadherins exhibit Ca^{2+}-dependent adhesion: the removal of Ca^{2+} from the extracellular solution results in a loss of adhesion. Thus, it is possible that changes in extracellular Ca^{2+} associated with synaptic activity may alter cadherin-cadherin interactions. We are attempting to visualize cadherin-cadherin associations at cell-cell junctions with the eventual goal of monitoring hippocampal synapses during synaptic activity. This will enable us to determine if synaptic activity and plasticity affects cadherin dynamics and synaptic structures. To visualize cadherins in living cells, we have expressed multiple E-cadherin constructs with ECFP or EYFP green fluorescent protein (GFP) variants or HA or FLAG epitope tags inserted on a flexible linker in several different non-structural loops at various sites on the extracellular domain in mouse L cells. The different constructs can be expressed in different populations of L cells, which are known to express no or very low levels of cadherins. To use the GFP variants as an example, ECFP can act as a fluorescence resonance energy transfer (FRET) donor for EYFP and FRET will be used to visualize the homophilic interactions between cadherins on adjacent cells. Sindbis viral vectors have been prepared for expression of these constructs in neurons. Cadherin containing one GFP variant (for example) will be expressed in presynaptic neurons while cadherin containing the other GFP variant will be expressed in postsynaptic neurons. FRET will then be used to detect these exogenous cadherins and their homophilic interactions between pre- and postsynaptic cells in differing conditions. In particular, we will examine the effects of synaptic activity and varying extracellular calcium concentrations on cadherin-cadherin dynamics. Surprisingly little is known about the cadherin homophilic interaction; hopefully this approach will not only allow us to learn something about cadherin's role at the synapse but also shed some light on the basic nature of this interaction.

350. Regulation of β-catenin-cadherin affinity by Cdk5 in response to depolarization in hippocampal neuron

Sachiko Murase

Morphological changes at synapses are one mechanism to achieve long-term synaptic plasticity. Activity-dependent changes at synapses are likely orchestrated by cell adhesion molecules (CAMs), which mediate synaptic connections and interact with the cytoskeleton. Cadherins are a family of CAMs, present at synaptic sites and involved in long-term potentiation. A cytosolic protein, β-catenin is known to mediate the interaction between cadherin and actin filaments, which is important for cadherin adhesion. We have recently demonstrated that depolarization increases β-catenin-cadherin affinity, and drives pines, which promotes structural change to enhance synaptic function (Murase *et al*., 2002). Since Cdk5/p35 kinase activity had been reported to decrease the affinity of β-catenin for cadherin in developing cortical neurons (Kwon *et al*., 2000), we investigated the possible involvement of Cdk5 in depolarization-induced modulation of β-catenin-cadherin interaction. In hippocampal neurons, blocking Cdk5

activity resulted in a redistribution of EGFP-catenin from dendritic shaft to spines where cadherins are highly concentrated. The amount of β-catenin co-immunoprecipitated with cadherin was also significantly increased by the inhibition of Cdk5. The redistribution of β-catenin induced by a specific inhibitor, roscovitine, was similar to that induced by depolarization. Pre-depolarization completely blocked the effect of roscovitine, whereas pre-incubation of roscovitine did not block the depolarization-induced redistribution, suggesting the inhibition of Cdk5 is a part of the events induced by depolarization. Accordingly, depolarization caused a significant decrease in Cdk5 kinase activity, without changing the level of either Cdk5 or p35, suggesting that the proteasome pathway is not involved. Interestingly, the redistribution induced by Cdk5 inhibitor was completely blocked by a tyrosine phosphatase inhibitor, orthovanadate, and also by point mutations of β-catenin Tyr-654 to either Glu or Phe. Roscovitine treatment did not affect the amount of cadherin co-immunoprecipitated with anti-GFP antibody from the EGFP-Y654F-β-catenin-expressing hippocampal slices, whereas a significantly increased amount of cadherin was co-immunoprecipitated by roscovitine treatment when EGFP-β-catenin-expressing slices were used. These results suggest that Cdk5 activity regulates the affinity of β-catenin for cadherin by changing phosphorylation level of β-catenin Tyr-654.

351. Cortical-hippocampus circuitry - memory and behavior

Miguel Remondes

The most studied hippocampal input is the so-called tri-synaptic pathway. Axons from the entorhinal cortex (EC) layer II make synapses onto the dentate granule cells; these send axons (mossy fibers) to CA3 pyramidal cells in the hippocampus. From CA3, axons (Schaffer collaterals) make synapses onto CA1 pyramidal cell dendrites. Finally, the axons from CA1 pyramidal cells carry the major output from the hippocampus onto the deep layers of EC, completing the cycle. This tri-synaptic circuit is thought to sub-serve most of the hippocampus-dependent mental functions. There exists, however, another cortex-hippocampus input that has not received much attention. This input leaves EC layer III cells and makes synapses directly onto CA1 pyramidal cells, by-passing the above mentioned tri-synaptic pathway. Recently, we communicated the results of an extensive *in vitro* physiology study that showed that this pathway - temporoammonic pathway (TA) - has the capacities for autonomous encoding of information in the cortico-hippocampal memory circuit. We further showed that it can act as a temporal discriminator of the tri-synaptic pathway input and plasticity to CA1. Lastly, we have shown that these short- and long-term modulation properties are themselves subject to long-term bi-directional changes (plasticity). We are now examining its role in memory encoding and retrieval in the awake, behaving rat. In other words, we are asking the following question: Can a rat perform spatial orientation and spatial memory encoding, without the TA pathway? In order to answer this question, we are suppressing the TA pathway through electrolytic lesions and assessing the rat's performance in a well-documented spatial memory task – the Morris Water Maze. We hope to clarify whether or not this pathway participates in acquisition, storage and/or retrieval of spatial information in the brain.

352. The neural bases of learning and memory in humans

Jessica Edwards

The ability for humans to form new memories, recall previously stored memories and consolidate new information is one of the hallmark features of human cognition. At present, the mechanisms by which memories are formed and recalled in the brain are not well understood. Our current understanding of human memory has been advanced primarily by lesion studies and neuropsychological testing. Such observations have strongly implicated the hippocampus and limbic structures in the formation new memories and the recall of these memories, especially verbal memory, visual memory, and emotional memories. Nonetheless, these observations have not been able to establish a mechanistic description of how the limbic system accomplishes these learning and memory tasks. In contrast, our knowledge about the neuronal mechanisms underlying learning and memory has been largely advanced through animal research. By direct measurement of neuronal activity using electrodes in animals, tissue preparations from animals, and cells grown in culture, we have learned that correlated activity between two interacting neurons can result in long-term changes in the responses of these individual neurons. This long-term potentiation (LTP) or long-term depression (LTD) is believed to represent the fundamental mechanism for memory formation. However, cognitive limitations exist in using animal studies to explore learning and memory.

We have the rare opportunity to record neuronal activity at the single unit level from alert, behaving humans. Patients suffering from medically intractable epilepsy are resistant to drug therapies that are traditionally used for seizure control. When the epileptogenic focus is well localized, removal of the small portion of offending tissue can cure a patient of epilepsy in over 85% of cases. To localize the area for surgery, patients are implanted with up to twenty electrodes, including several microwire electrodes (40 micron, Pt/Ir wires) in the hippocampus and amygdala. Because electrodes are being placed for clinical diagnostic purposes, an opportunity exists to simultaneously record individual neuronal activity. The duration of the medical procedures allows us to monitor the neuronal activity of cells in the hippocampus and amygdala for up to one week. Using a battery of neuropsychological tests, we are able to examine rapid learning and declarative memory. Tests we are currently using include the Recognition Memory Task, a Continuous Visual Memory Task, Verbal Paired Associates and others. We also use a virtual Water Maze, a joystick-operated

simulation of the Morris Water Maze task, to test object-cued place memory. Analysis of these data may be able to demonstrate a direct relationship between hippocampal activity and memory formation in humans. Further, we hope to examine the correlation between local field potentials (EEG) and single-unit activity. The ability to record neuronal activity in the human brain in response to behavioral testing should provide a truly unique opportunity to synthesize our knowledge about human memory function with our knowledge about the mechanism of neuronal activity.

Publications

Aakalu, G., Smith, W.B., Nguyen, N., Jiang, C. and Schuman, E.M. (2001) Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* **30**:489-502.

D'Apuzzo, M., Mandolesi, G. and Schuman, E.M. (2001) Abundant GFP expression and synaptic plasticity following *in vivo* Sindbis virus injection. *J. Neurophysiol.* **86**:1037-1042.

Jiang, C. and Schuman, E.M. (2002) Local protein synthesis. *Trends Biochem. Sci.* **27**:506-513.

Murase, S. and Schuman, E.M. (2002) Cdk5 regulates activity-induced changes in cadherin-β-catenin affinity. In review.

Murase, S., Mosser, E. and Schuman, E.M. (2002) Depolarization drives β-catenin into neuronal spines promoting changes in synaptic structure and function. *Neuron* **35**:91-105.

Remondes, A.M. and Schuman, E.M. (2002) Direct cortical input modulates spiking and plasticity in CA1 pyramidal neurons. *Nature* **416**:736-740.

Remondes, M. and Schuman, E.M. (2002) Properties of early- and late-phase LTP at temporoammonic-CA1 synapses. In review.

Smith, W.B., Aakalu, G.N. and Schuman, E.M. (2001) Local protein synthesis. *Curr. Biol.* **11**:R901-903.

Steward, O. and Schuman, E.M. (2001) Protein synthesis at synaptic sites on dendrites. *Ann. Rev. Neurosci.* **24**:299-325.

Tang, S., Meulmans, D., Vasquez, L., Colaco, N. and Schuman, E.M. (2001) A role for staufen in the delivery of RNA to neuronal dendrites. *Neuron* **8**:463-475.

Tang, S., Reis, G., Kang, H. Gingras, A.C., Sonenberg, N. and Schuman, E.M. (2002) A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *PNAS* **99**:467-472.

Professor of Biology: Kai G. Zinn
Postdoctoral Scholars: Mili Jeon, Kaushiki Menon, Rachel Kraut (Papan), Anuradha Ratnaparkhi, Nina Sherwood
Graduate Students: Bruce Burkemper, Anna Salazar
Research and Laboratory Staff: Elena Armand, Lakshmi V. Bugga, Violana Nesterova, Cindy Shih
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Summary: Our overall focus is on the molecular mechanisms of axon guidance and synaptogenesis in *Drosophila*. Our approach combines genetics, molecular biology, biochemistry, and cell biology. We are especially interested in cell-surface and signal-transduction proteins that function in growth cones, presynaptic terminals, and their postsynaptic partners.

Receptor tyrosine phosphatases and axon guidance: Genetics of receptor tyrosine phosphatases. In the 1990s, we showed that four 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 are likely to 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.

We have performed a detailed characterization of the genetic interactions among five of the six RPTPs. We find that each growth cone guidance decision in the neuromuscular system has a requirement for a unique subset of RPTPs. 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 [Sun *et al*. (2001) *Mol. Cell. Neurosci.* **17**:274-291].

Double mutants lacking two RPTPs (DPTP10D and DPTP69D) have a unique and highly penetrant phenotype in which longitudinal axons are routed across the midline of the embryo [Sun *et al*. (2000) *Development* **127**:801]. The double *Rptp* combination interacts with *robo*, *slit*, and *comm*, a set of mutations affecting a pathway controlling repulsion of longitudinal axons from the midline. These results indicate that DPTP10D and/or DPTP69D may be positive regulators of signaling through the Robo family of receptors.

By analyzing genomic sequences, we identified a new RPTP, DPTP52F, which contains five FN3 repeats and a single PTP domain and is selectively expressed in the CNS. We examined the role of DPTP52F by inhibiting its expression using dsRNA (RNAi) injection into embryos and by identifying EMS mutations in its gene. *Ptp52F* single mutants cause motor and CNS axon guidance phenotypes, and *Ptp52F* interacts genetically with *Ptp69D*, *Dlar*, and *Ptp10D* [Schindelholz *et al*. (2001) *Development* **128**:4371-4382]. We have also recently obtained mutants lacking expression of the sixth (and final?) RPTP, DPTP4E, and are investigating their phenotypes.

Searching for RPTP substrates and interacting proteins. We are taking several approaches to address this problem. It is difficult to identify PTP substrates biochemically because PTPs usually do not display strong specificity *in vitro*. We previously identified a potential DPTP10D substrate, Gp150, using biochemistry [Fashena and Zinn (1997) *Mol. Cell. Biol.* **17**:6859]; but subsequent genetic studies (Q. Sun, unpublished) indicated that Gp150 was unlikely to be important for axon guidance in the embryonic CNS.

One current approach is to perform yeast two-hybrid screens with 'substrate-trap' versions of DPTP10D, DPTP69D, and DPTP99A. We introduced a constitutively activated chicken Src tyrosine kinase into the yeast together with the PTP 'bait' constructs, in the hope that it would phosphorylate relevant substrate fusion proteins made from cDNA library plasmids. The screen should also recover non-substrate interacting proteins that may form complexes with the RPTPs *in vivo*. We have identified several classes of clones whose interactions with the substrate-trap RPTPs are dependent on coexpression of the tryosine kinase, suggesting that they may be substrates. We are currently analyzing these further to determine which ones are likely to be of interest for the future (see Bugga abstract).

We have also purified a substrate-trap DPTP69D protein from embryos in which its overexpression causes axon guidance phenotypes (this line was made by Paul Garrity's group at MIT). The implication of the phenotype is that the substrate-trap protein is sequestering proteins that participate in guidance *in vivo*; accordingly, it will be of interest to determine what might be complexed to this protein in overexpression embryos. We hope to analyze the proteins in the putative DPTP69D complex by cutting bands from a gel and determining portions of their sequences. These experiments are being done together with Dr. Peter Snow, the Director of the Protein Expression Facility at Caltech (see Burkemper abstract).

Searching for RPTP ligands, and development of a new method for identification of *Drosophila* cell-surface proteins. We have conducted several mammalian COS cell expression screens to attempt to identify ligands and/or coreceptors for the fly neural RPTPs, so far without success. This has been a major problem in the RPTP field; indeed, *in vivo* ligands for RPTPs 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.

Our current approach to identifying ligands is based on our observation (Aloisia Schmid, unpublished; see Zinn abstract) that fusion proteins in which the extracellular domains of RPTPs are joined to human placental alkaline phosphatase (AP) can be used to stain live *Drosophila* embryos. Each of four fusion proteins (DLAR-AP, DPTP69D-AP, DPTP10D-AP, DPTP99A-AP) binds in a specific manner to the embryonic CNS. Most of the observed staining is on CNS axons. We are now screening a 'deficiency (Df) kit' of ~200 fly lines, each of which lacks a specific region of the genome, by staining homozygous Df embryos from each line with each of the fusion proteins. This method should identify the genomic regions encoding each of the RPTP ligands. We have already identified Dfs that contain genes required for DPTP69D-AP and DLAR-AP staining (see Zinn abstract). We are currently using overlapping Dfs and RNAi methods to identify the single genes that confer specific staining.

We are also developing a novel method for expressing the entire repertoire of fly cell-surface proteins on the surfaces of transfected mammalian cells. Cells expressing this collection of cell-surface proteins can be screened using RPTP-AP fusion proteins in order to identify and characterize ligands. We will also generate monoclonal antibodies (mAbs) to these cell-surface proteins, and we have developed an approach that should allow us to directly clone the genes encoding the proteins recognized by these mAbs without any biochemical purification or screening of expression libraries. These genes and mAbs should be of interest in understanding axon guidance as well as other processes that occur during fly development.

Screens for genes controlling axon guidance and synaptogenesis. An overexpression/misexpression screen for genes that regulate axon guidance and synaptogenesis in the larval neuromuscular system. The 32 motor neurons in each hemisegment of a *Drosophila* embryo innervate 30 muscle fibers, and each motor axon extends along a stereotyped route and always targets the same fiber. Motor growth cones reach their muscle targets during late embryogenesis and then gradually mature into presynaptic terminals. 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. The pattern of Type I neuromuscular junction (NMJ) synapses in the third instar larva is simple and highly stereotyped, with boutons restricted to specific locations on each muscle fiber.

Although these features should make the third-instar NMJ pattern an ideal system in which to perform genetic screens to identify molecules involved in the assembly of synaptic circuits, there are problems in the execution of such screens. Anatomical screens typically require dissection and antibody staining of larvae from each mutant line, which is very time-consuming. Also, due to 'genetic redundancy', single loss-of-function (LOF) mutations in genes involved in axon guidance or synaptogenesis often do not produce strong neuromuscular phenotypes.

In order to perform a forward screen for axon guidance and synaptogenesis phenotypes that avoids the problems with LOF screens of dissected animals described above, we devised and executed a gain-of-function (GOF) screen of live larvae [Kraut *et al*. (2001) *Curr. Biol.* **11**:417-430]. We used the 'EP' transposable element to drive high-level expression of genes in motor neurons. This overexpression strategy was chosen in order to sensitize the screen and minimize the problem of genetic redundancy. Work on transcriptional regulators, neural cell-surface proteins, and signaling molecules has shown that pan-neuronal overexpression can produce strong effects on motor axon guidance and/or synaptogenesis, even when LOF mutations produce only weak phenotypes that would be missed in a screen. Genetic redundancy may allow growth cones to identify their targets and differentiate into synapses in the absence of a pathway involved in response to a specific adhesive or repulsive cue. An excess of signaling through a response pathway, however, can upset local balances between attraction and repulsion and cause errors in pathfinding and/or synaptogenesis.

It is important to note that overexpression/ misexpression screens are inherently risky, in that high-level expression of a gene in a particular cell type may produce a phenotype even when the gene is not normally involved in development of that cell type. We have thus developed strategies for rapidly sorting isolated genes to identify those that participate in neuromuscular development in wild-type animals.

Our screen identified 41 'known genes' (those with published mutant alleles) and 35 'new genes' for which high-level neuronal expression produces axonal/synaptic phenotypes. We assembled published phenotypic data on the 'known genes', and examined larval neuromuscular LOF phenotypes for some of them ourselves. These results showed that at least 3/4 of the 'known genes' are important for nervous system development or function in wild-type flies. An analysis of homology relationships displayed by the 'known gene' and 'new gene' sets suggests that most of the 'new genes' will also have neural LOF phenotypes. The products encoded by the 76 genes identified in our screen include kinases, protein and lipid phosphatases, GTPases, guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), ATPases, cell-surface receptors, RNA-

binding proteins, transcriptional regulators, and a variety of other molecules likely to be involved in protein trafficking, modification, and degradation. ~85% of the 'new genes' examined thus far are expressed in the wild-type CNS, suggesting that they normally function during nervous system development.

To begin to determine whether the 'new genes' have neural LOF phenotypes, we homozygosed their EP elements, then examined motor axons and synapses in mutant larvae by antibody staining. P elements located in the 5' ends of genes often disrupt promoters and thus reduce gene expression. Many EPs are homozygous lethal, suggesting that they do affect expression of adjacent genes. For 'new genes' where no phenotype was observed for the homozgous EP insertion, we began generating LOF phenotypes using RNAi methods. In *Drosophila*, RNAi is usually performed by injecting dsRNAs for a portion of an mRNA into syncitial blastoderm embryos, allowing these to develop, then examining phenotypes in embryos or hatched larvae [Schmid *et al*. (2001) *Trends Neurosci*., in press]. To streamline the process of searching for RNAi phenotypes, we developed a protocol in which we inject dsRNAs for each gene into Elav-GAL4, UAS-GFP embryos, then pick hatched larvae that contain coinjected blue dye in their guts and allow them to develop to third instar. We can then directly examine the neuromuscular system of these live larvae as we did in the original screen. This procedure eliminates nonspecific phenotypes resulting from injection damage, because damaged embryos usually do not hatch, and those larvae that do hatch do not feed and die quickly. Since we do not have to dissect and stain the larvae with antibodies, we can examine a large number of hemisegments in order to quantitatively assess phenotypes. Thus, even if an observed phenotype is of low penetrance, we can still detect and evaluate it.

We have initiated further study of a number of genes identified in the screen that are defined by existing mutations. *pumilio* (*pum*) encodes an RNA-binding protein that shuts down translation of specific mRNAs by binding to their 3' untranslated regions. We found that Pum protein is expressed in a small subset of CNS neurons, and *pum* LOF mutations alter motor axon guidance in the embryo. Pum protein is also localized to NMJs in third instar larvae, and is primarily postsynaptic. *pum* LOF larvae have NMJs that do not grow in a normal manner. Postsynaptic Pum regulates expression of a translation initiation factor, eIF-4E, which is localized to postsynaptic aggregates; thus, in *pum* mutant larvae we observe a dramatic increase in the number and size of eIF-4E aggregates. Pum protein binds directly to the 3'UTR of eIF-4E mRNA (see Menon abstract).

Robo2 is a cell-surface protein that mediates growth-cone repulsion from sources of its ligand Slit. We find that Robo2 overexpression alters the migration of sensory neuron cell bodies in the peripheral nervous system (PNS), and LOF mutations in Robo family genes also affect PNS cell migration (see Kraut abstract 355).

'New' genes identified by the screen include a number of molecules involved in protein trafficking in neurons. We have investigated two of these thus far. Beached is a huge protein that is closely related to the human protein whose loss causes Chediak-Higashi syndrome, a lethal disease affecting lysosomes and related organelles (see Kraut abstract 356). Gartenzwerg (Garz) is a Sec7-related GEF that is orthologous to a plant GEF that regulates cell polarity during development (R. Kraut, C. Shih, unpublished).

An AAA-ATPase that regulates axon guidance across the midline. We also performed an EP screen in which we generated 5000 new EP lines and crossed these to the Elav-GAL4 driver, selecting those that produced driver-dependent. One EP, T32, produces a dramatic overexpression phenotype in which the CNS is narrowed and axons abnormally cross the midline. This EP is inserted immediately upstream of a gene encoding an AAA-ATPase that is the ortholog of human spastin, a gene mutated in the disease autosomal dominant spastic hemiplegia. AAA-ATPases are a class of proteins that form multimeric complexes that regulate a variety of protein trafficking events within the cell, including vesicle sorting, protein degradation, and microtubule dynamics. There are about 30 such proteins encoded in the fly genome. We made LOF mutations in the T32 (*spastin*) gene, and found that these produce weak motor axon phenotypes. T32-LOF homozygotes are viable but display abnormal behaviors. Our current focus is on examining T32's role in axon guidance via genetic interaction studies and yeast two-hybrid screens to identify the other components of the pathways in which it acts (see Sherwood abstract).

Screening for genes involved in targeting of motor axons to embryonic muscles and olfactory receptor axons to the antennal lobe. We are devising screens to identify genes which, when expressed in all muscle fibers, perturb targeting of specific motor axons without altering the muscles themselves (see Ratnaparkhi abstract). We are also using conceptually similar techniques to examine targeting of olfactory receptor axons to the glomeruli of the antennal lobe (see Salazar abstract). These screens are not yet ready for execution, because we need to build transgenic flies carrying novel gene expression systems in order to carry them out.

353. Two-hybrid screens for RPTP substrates

Lakshmi Bugga

To understand fully the function and regulation of *Drosophila* RPTPs, it will be necessary to identify the physiological substrates of the individual RPTPs. To isolate RPTPs in a complex with their target substrates, we are using yeast two-hybrid system. We are looking for proteins that interact with *Drosophila* neuronal RPTPs-10D, 69D, 99A and 52F. To attempt to achieve stable binding of the RPTPs to a tyrosine phosphorylated substrate, we have used 'substrate-trap' mutants of the RPTPs, which can bind to substrates but do not catalyze

dephosphorylation, instead remaining bound to substrate in a stable complex.

We constructed plasmids encoding GAL4 DBD/RPTP bait proteins and introduced them into yeast together with fly cDNA libraries encoding GAL4AD-cDNA fusion proteins and a plasmid containing a constitutively active form of chicken c-Src, driven by a constitutive yeast promoter. The bait (with or without src) and prey (cDNA) hybrid proteins are transformed into separate yeast mating strains (A and alpha) that contain three reporter genes-Adenine, Histidine, and LacZ, and also an auxotrophic marker. Positive interactions are detected by selecting on plates lacking the auxotrophic marker and screening for reporter expression.

Our screen with all four DPTPs identified six genes that interact specifically with a given DPTP. Four of these genes encode potential substrates based on Src dependence. Three of the six are known genes: Tartan (a cell adhesion molecule expressed in embryonic CNS and PNS); cysteine string protein (a chaperone protein expressed in larval neuromuscular junction and adult brain); and Xmas-2 (an RNA binding protein that is involved in spermatogenesis, oogenesis and embryogenesis). Of the three unknown genes, one is rich in proline residues and also has proline motifs that are known to bind to SH3 domains. RNA *in situs* with this gene showed expression in the embryonic CNS. We are currently testing these interactions *in vitro* by transient transfection experiments with *Drosophila* cell line-S2 cells, and examining the RNA expression patterns for the new genes using *in situ* hybridization.

354. Studies of proteins purifying with a 'substrate-trap' version of DPTP69D

Bruce Burkemper, Peter Snow

We have purified a "substrate trap" version of DPTP 69D from transgenic flies and isolated several proteins that co-purify with it. DA3 has a point mutation in an aspartic acid residue that causes it to remain bound to substrate. This is likely the basis for the axon guidance phenotype seen in flies expressing DA3. We purified DA3 from transgenic embryos overexpressing the mutant protein in the CNS. An embryo lysate preparation is applied to a ConA column to enrich for surface proteins, followed by an anti-DPTP69D column. Bands purified from the DA3 embryos that were not present in negative control preparations (wild-type purified with anti-DPTP69D and DA3 applied to a control antibody column) were selected for sequencing. The first two proteins so identified are members of a class of proteins called protein disulfide isomerases (PDIs). These proteins assist in protein folding—making, breaking and rearranging disulfide bonds as a protein matures and assumes its final conformation. In light of this finding, one way to interpret the DA3 phenotype is to speculate that misfolded PTPs fail to make it out to axons. However, staining with PTP antibodies has shown no evidence for mislocalization of PTPs in DA3 embryos. Furthermore, the PTP's catalytic domain is in the cytoplasm and PDIs are usually found in the ER lumen; these disparate cell localizations would preclude an interaction. A third protein of 220 kD was present in both DA3 and wild-type DPTP69D preparations, but absent from control antibody purifications. The specificity of the interaction encouraged us to pursue this protein as one that may have a meaningful interaction with DPTP 69D, even if it proved not to be a substrate. We used mass spectrometry techniques to identify this protein as Zipper. Zipper, also known as nonmuscle myosin II, or mysosin heavy chain (MHC), plays a role in several developmental processes. These include axon patterning, dorsal closure, and imaginal disc development. We are currently using genetic approaches to determine whether DA3 phenotypes arise from alterations in Zipper function.

355. The Robo2 axon guidance receptor also guides sense organ cell migration

Rachel Kraut (Papan)

In our overexpression screen for genes involved in motorneuron axon guidance, we discovered serendipitously that Robo2, a well-known axon guidance receptor, affects the migration of sense organs in the periphery. *Robo2* and two related genes, *Robo1* and *Robo3*, make up a family of receptors for the repulsive secreted signal *slit*, which is found at the CNS midline. The Robo receptors are expressed in overlapping subgroups of longitudinal axons and prevent these axons from crossing the midline. When overexpressed in PNS neurons, Robo2 causes a failure of some sense organs, the stretch receptors, to migrate as usual. Normally, all stretch receptors in abdominal segments begin life with their progenitors at a dorsal position, and as they develop, they rotate and move ventrally. In robo2-overexpressing embryos, these organs are caught in varying degrees of attempted migration/rotation. This does not happen upon overexpression of the other robo molecules, or the slit ligand. Perplexingly, the *robo2* loss-of-function phenotype does not affect chordotonal morphology. However, *robo2* and *robo1* mutations together cause excessive migration in a certain subpopulation of stretch receptors, which can be interpreted as being complementary to the "anti-migratory" gain-of-function phenotype of *robo2*. The robo ligand slit also affects stretch receptor migration: in *slit* mutants, certain stretch receptors migrate ectopically, similarly to the *robo1,2* loss-of-function. This indicates a positive interaction between the slit ligand and the robo receptor(s), as is the case at the midline. In contrast, other stretch receptors fail to migrate, similarly to the *robo2* gain-of-function. This indicates a negative interaction between slit and robo2, unlike that at the midline. Consistent with these results is the expression of all of these genes (*robo1,2,3* and probably *slit*) in chordotonal organs, with *robo2* being preferentially expressed in organs that normally do not migrate. We plan to investigate the possibility that *slit* can interact with the different robo receptors either in the *cis* or *trans* orientation, and that this orientation, along with the

type of receptor, and the relative levels of ligand vs. receptor, can determine the sign of the interaction.

References

Kidd, T. *et al.* (1999) *Cell* **96**:785-794.
Kurant, E. *et al.* (1998) *Development* **125**:1037-1048.
Rajagopalan, S. *et al*. (2000) *Neuron* **28**:767-777.
Rajagopalan, S. *et al.* (2000) *Cell* **103**:1033-1045.
Simpson, J.H. *et al.* (2000) *Cell* **103**:1019-1032.
Simpson, J.H. *et al.* (2000) *Neuron* **28**:753-766.

356. New pathways in axon guidance: The interface between endocytosis and lipid signaling

Rachel Kraut (Papan)

Previously, we carried out an overexpression screen to identify genes which when expressed at high levels in the nervous system lead to defects in the pattern of neuromuscular connectivity in the larva. About 70 genes had some effect on motoraxon navigation and/or synapse formation, and about half were novel.

Surprisingly, a disproportionate number of the genes we identified turned out to be potentially involved in endocytosis. One gene we are pursuing from our screen, called *beached*, may function in both endocytic and lipid-signaling pathways, but this is still unclear. Beached is a very large protein with a FYVE domain that localizes proteins to PI(3)P, found exclusively in endosomal membranes. It also contains a BEACH domain (for BEige And Chediak Higashi syndrome), which is found in several proteins that have been implicated in endolysosomal trafficking in function and may be linked to sphingomyelin breakdown, which produces the second messenger ceramide. Ceramide is not only a second messenger in its own right, serving to activate Phospholipase D (PLD) and apoptosis in some cell types; it is also an intermediate in the production of Sphingosine-1-Phosphate (S-1-P). S-1-P is a good candidate for a lipid signal in growthcone guidance, since it can be secreted, and affects rho-family GTPases and PLD signaling in cells to which it is applied, which in turn impinge upon the actin cytoskeleton, effecting morphological changes such as lamellipodia and spreading.

RNAi for *beached* gives a highly specific pathfinding error in one of the motornerve bundles. Consistent with this, *beached* is specifically expressed in a subset of CNS axons during axon outgrowth. Interestingly, the protein localizes to what appear to be vesicles, but the identity and subcellular location of these is unknown.

357. Pumilio, a postsynaptic protein regulating synaptic morphology and expression of a translation initiation factor

Kaushiki Menon

We identified *pumilio* (*pum*) in our overexpression screen for larval synaptogenesis phenotypes, and then characterized the phenotypes of *pum* LOF mutants. *pum* larvae have abnormal NMJs with reduced synaptic span and fewer boutons than normal. Our results suggest that *pum* regulates the maturation of new synaptic boutons during larval growth. Pum protein is localized to the postsynaptic side of the NMJ, and our preliminary results suggest that expression of *pum* cDNAs in muscle is sufficient to rescue the synaptic phenotypes. *pum* is known to function during early embryonic development by binding to the 3'UTR of *hunchback* mRNA and repressing its translation in the abdominal region. We speculated that synaptic Pum might regulate eIF-4E, a translation initiation factor that has been shown to be localized to postsynaptic aggregates. We have now shown that this is in fact the case: in *pum* mutants, the number and size of eIF-4E aggregates are greatly increased. In collaboration with Robin Wharton's group at Duke University, we have shown that Pum protein binds very tightly to the 3' UTR of eIF-4E mRNA, indicating that this initiation factor is likely to be a direct target for Pum translational repression. eIF-4E regulation is likely to be part of the mechanism by which *pum* regulates synaptic structure and function.

358. A misexpression screen to identify target-derived molecules that direct appropriate innervation of larval body wall muscles

Anuradha Ratnaparkhi

The process of target recognition by motor axons can be divided into a two-step process: the first step involving defasciculation of the motor axons from the motor nerve at specific choice points. RPTP's and other cell-surface molecules such as Fasciclin II are known to play a role in regulating this process (REFS). The second step involves recognition of the specific muscle target. This process must require muscle-derived factors that are crucial for recognition by the motor axons. So far only two target-specific genes, *beaten path* and *side step*, have been identified that regulate branching and innervation of muscles. In order to identify muscle-derived factors essential for the process of innervation and synaptogenesis, we propose to initiate a muscle misexpression screen using the EP modular overexpression system. In this screen we intend to use a modified EP that allows bi-directional gene expression. This would thus enable us to screen twice the number of genes as compared to the original EP. In order to facilitate visualization of the muscle as well as the nerves simultaneously during the screen, we intend to use two modular expression systems: the widely-used UAS-GAL4 system to drive the EP plus a reporter like UAS-dsRed in the muscle; the LEX-GAD system to drive expression of EGFP in the axons. For this purpose, we will need to make transgenic flies expressing LexA under the control of the Elav promoter (Elav-LexA) and also flies carrying the LexA binding sequence tagged to EGFP. This screen will be conducted by crossing new EP transposants to Elav-LexA and OR promoter drivers (see Salazar abstract). In this way, we can simultaneously screen for genes regulating targeting of axons to muscles and to glomeruli of the antennal lobe.

359. Genetic analysis of targeting of sensory neuron axons to the antennal lobe

Anna Salazar

Due to the recent elucidation of a family of odorant receptors in *Drosophila melanogaster*, further efforts to gain insight into the topic of olfaction have progressed into studies of *Drosophila*, whose genetic capabilities make it a model organism for this type of study. Olfactory sensory neurons in *Drosophila* are housed in sensory hairs, called sensilla, which are located in the two olfactory organs of the adult fly, the third antennal segment and the maxillary palp. It has been shown that the axons of antennal sensory neurons expressing a particular olfactory 7-helix receptor all project to a single glomerulus in the antennal lobe. We have obtained a line from Dr. Leslie Vosshall in which an olfactory receptor promoter drives GAL4. The same strain also contains a UAS-GFP that is presynaptically localized. In adults from this line, GFP is observed within a single large glomerulus on each side of the antennal lobe. We are also making olfactory receptor promoter-GAL4 transgenic lines with other promoters. We wish to use these fusions to drive GFP expression in antennal sensory neurons, while simultaneously driving expression of random genes in the antennal lobe target region using the EP system. We will then generate new EP transposants and screen them for mistargeting phenotypes. This screen should allow identification of genes controlling appropriate targeting of the axons of sensory neurons. To drive sufficient GFP expression, we need to create a new amplification system that is independent of the GAL4 system used to drive expression from the EP elements. We are currently developing a system based on an activation protein, LexGAD, that binds to LexA sites inserted upstream of a basal promoter (see Ratnaparkhi abstract). Finally, we are examining the role of Or 83b, an olfactory receptor that is expressed in all antennal sensory neurons.

360. *DSpastin,* the fly ortholog of a human spasticity gene

Nina Tang Sherwood

We identified the *Drosophila* ortholog of the human *spastin* gene, *Dspastin* (line EPT32), through a neuronal overexpression screen for novel molecules mediating axon guidance in the fly embryo. Mutations in human *spastin* (also called SPG4) underlie the predominant form of autosomal dominant hereditary spastic paraplegia (AD-HSP). This disease is characterized by progressive spasticity and weakness of the lower limbs due to the neurodegeneration of descending corticospinal tracts.

Spastin encodes a novel member of the large and functionally diverse AAA ATPase family of proteins. Recent experiments using exogenous expression of human Spastin in cell lines (Errico *et al*., 2002) suggest that the protein may function by associating with microtubules via its N-terminal region and disassembling them in an ATP-dependent manner. This is reminiscent of the closely-related AAA ATPase p60 katanin, which forms hexameric complexes in order to sever microtubules at the centrosome and mitotic spindle poles.

A polyclonal antibody generated against *Dspastin* suggests that in embryos the protein is most highly expressed in the CNS; specifically, in the nuclei of four cells (two bilaterally symmetric sets) per hemisegment. One set of cells is the RP2 motoneurons, which innervate muscles 2, 9, and 11 via the intersegmental nerve (ISN) pathway. The other set of cells lies more dorsally, and may be glial in identity.

We have generated four imprecise excision mutation lines, three of which are partial deletions of the region encoding the N-terminal (non-catalytic) region of *Dspastin*, and one in which the entire gene is excised. RT-PCR experiments show that mRNA, and thus most likely truncated *Dspastin* protein, is still synthesized in the partial deletion lines, indicating that these mutants are probably hypomorphs. All four lines are homozygous viable, but in the complete deletion line homozygous adults are found at greatly reduced frequency and late pupal lethality is observed. Furthermore, young adult flies with even a partial deletion of *Dspastin* are slow moving and poor fliers. Together with the CNS expression pattern, these data suggest that a deletion in *Dspastin* may compromise motor ability in these flies, to the extent that flies completely lacking the protein have difficulty enclosing. We are currently examining the complete deletion line for defects in motoneuron pathfinding or synaptogenesis, particularly in the ISN, as well as characterizing *Dspastin* expression patterns more completely in the larval and adult stages.

Reference

Errico *et al.* (2002) *Hum. Mol. Genet*. **11**:153-163.

361. Searching for genes encoding RPTP ligands

Kai Zinn, Violana Nesterova, Cindy Shih

Aloisia Schmid, a former postdoc in our group, developed methods for staining live dissected embryos with RPTP-AP fusion proteins made in baculovirus by Peter Snow of the Protein Expression Facility. We have further optimized these methods and used them to screen a 'deficiency (Df) kit' of ~200 fly lines encompassing ~80% of the euchromatin to identify genes encoding proteins required for generation of these staining patterns. Such genes might encode the elusive RPTP ligands themselves, or might encode proteins essential for ligand synthesis and localization. DLAR-AP stains a pattern of CNS and peripheral glia as well as CNS axons. DPTP69D-AP, DPTP10D-AP, and DPTP99A-AP stain distinct patterns of CNS axons. To perform the screen, we first crossed GFP balancers into ~150 of the Df kit lines. We then collected stage 16 embryos from each lines, sorted GFP+ (Df heterozygotes and balancer homozygotes) and GFP- (Df homozygotes) and dissected ~5 embryos of each class for each fusion protein. We then stained these embryos with DLAR-AP and DPTP69D-AP fusion proteins in parallel. We have now identified a Df that eliminates staining of glia by DLAR-AP without affecting axonal staining.

Subsequent mapping with overlapping Dfs has localized the responsible gene to a small interval. More recently, we have found another Df that eliminates DPTP69D-AP axonal staining. We are currently mapping this putative gene further using overlapping Dfs.

Publications

Dubuque, S.H., Schachtner, J., Nighorn, A.J., Menon, K.P., Zinn, K. and Tolbert, L.P. (2001) Immunolocalization of synaptotagmin for the study of synapses in the developing antennal lobe of *Manduca sexta*. *J. Comp. Neurol.* **441**:277-287.

Kraut, R., Menon, K. and Zinn, K. (2001) A gain-of-function screen for genes controlling motor axon guidance and synaptogenesis in *Drosophila*. *Curr. Biol*. **11**:417-430.

Schindelholz, B., Knirr, M., Warrior, R. and Zinn, K. (2001) Regulation of CNS and motor axon guidance in *Drosophila* by the receptor tyrosine phosphatase DPTP52F. *Development* **128**:4371-4382.

Schmid, A., Schindelholz, B. and Zinn, K. (2002) Combinatorial RNAi: A method for evaluating the functions of gene families in *Drosophila*. *Trends Neurosci.* **25**:71-74.

Sun, Q., Schindelholz, B., Knirr, M., Schmid, A. and Zinn, K. (2001) Complex genetic interactions among four receptor tyrosine phosphatases regulate axon guidance in *Drosophila*. *Mol. Cell Neurosci*. **17**:274-291.

Integrative Neurobiology

John M. Allman
Richard A. Andersen
Christof Koch
Masakazu (Mark) Konishi
Gilles Laurent
Shinsuke Shimojo

Frank P. Hixon Professor of Neurobiology: John M. Allman
Graduate Students: Eliot Bush, Karli Watson
Research and Laboratory Staff: Atiya Hakeem, Nichole Tetreault

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Summary: We have shifted our focus of investigation to the neural bases of decision-making. We are studying a type of neuron, the spindle cells of anterior cingulate cortex, which are unique to humans and apes. The anterior cingulate cortex is strongly implicated in focused problem solving and the spindle cells may serve may serve an important role in this adaptive process in hominoids. Together with our economist colleagues we are also investigating through functional magnetic resonance imaging the brain structures involved in economic decision-making, which include the anterior cingulate cortex and Brodmann's area 10, which has undergone great expansion in human evolution. We are also studying the cognitive processes in decision-making in elderly subjects to determine how these change with age and experience. Finally we are continuing our comparative studies of retinal and brain structure and organization in an effort to find some of the basic principles underlying brain evolution.

362. Assessing the accuracy of meta-knowledge in neurologically healthy elderly subjects

Stephanie Kovalchik[1], Colin Camerer[2], David Grether[2], Charles Plott[2], John Allman

In our behavioral study, cognitive testing of 50 elderly subjects and 51 undergraduate students reveals that the older adults have significantly more accurate meta-knowledge (knowledge of their own knowledge) than the young adults. This suggests that general self-knowledge improves as healthy individuals age or individuals with enhanced meta-knowledge are more likely to survive to old age. We investigated these assessments in a population of carefully evaluated, neurologically healthy elderly subjects, who serve as controls for the Alzheimer's Disease Research Consortium (ADRC) studies being conducted in the Department of Gerontology at the University of Southern California. We presented to these subjects a battery of general knowledge questions, with binary answer choices, related to geography, history and popular culture and asked them to rate their confidence in the accuracy of their responses from 50%, the chance level, to 100% confidence. While performance at the 50% confidence level is similar for both groups, older subjects are significantly better calibrated for all of the other levels. For the 100%, 90%, 80%, 70%, and 60% levels, the average older adult performs with 94%, 66%, 62%, 71%, and 65% accuracy, respectively, while the young subjects only perform with an accuracy of 92%, 62%, 65%, 52%, and 53%. When assessing the response distribution behavior for the two samples, this evidence becomes even more compelling. Older individuals distribute their responses mainly between 100% and 50%, out of 991 questions answered 40% were at the 100% level, 27% were at the 50% level, and approximately 8% were for each of the other levels. In contrast to this behavior, students show a greater spread of response selections, 27% of 1016 responses were at the 100% level, 25% were at the 50% level, and approximately 12% percent were for each of the other levels. From this evidence, elderly subjects seem to evaluate their meta-knowledge in three ways: absolutely sure, in which they are in fact quite accurate; some knowledge, in which they are only about 66% accurate; and guessing, in which they perform just above chance. Unlike the older subjects, young adults seem to believe they can accurately discriminate their meta-knowledge between all six confidence categories. Despite this belief, the students, like the older adults, show only three categories of meta-knowledge discrimination.

[1]*SURF student, Caltech*
[2]*Economics Professors. Humanities and Social Sciences, Caltech*

363. The distribution and size of retinal ganglion cells in prosimian primates: Implications for the evolution of sensory systems

Nicole Tetreault, Atiya Hakeem, John Allman

In previous work we mapped the size and distribution of retinal ganglion cells in flattened retinal whole mounts in the dwarf lemur, *Cheirogaleus medius*, and the Philippine tarsier, *Tarsius syrichta*. We have extended this comparison to the mouse lemur, *Microcebus murinus,* and also compared peak retinal ganglion cell density and other retinal parameters using published data for a wide variety of mammals. These data show that the ganglion cell density in the central retina is much lower in *Cheirogaleus* than in other prosimian primates (tarsiers, galagos and mouse lemurs). Dwarf lemurs eat mainly fruit and occasional crawling insects, whereas the other prosimians have a much larger proportion of insects in their diet and capture flying insects. Using volumetric data, we found that the olfactory bulb occupies a larger percentage of the total brain volume in dwarf lemurs as compared to mouse lemurs, which is consistent with the larger, furless rhinarium in dwarf lemurs. The dwarf lemur's large olfactory bulb is not the consequence of allometric variation. We conclude that the primitive condition for primates represented by tarsiers, galagos, and mouse lemurs involves high acuity, the ability to catch fast-moving prey, and a reduced emphasis on olfaction. Dwarf lemurs may be secondarily specialized for olfactory-guided fruit eating, reduced capacity to capture insects and reduced visual acuity.

364. Single-unit electrophysiology of the anterior cingulate cortex of human subjects

Karli Watson, Adam Mamelak[1], John Allman

In collaboration between Caltech and Huntington Hospital, we record from single neurons of the anterior cingulate cortex of human subjects. The data we gather is collected from patients with intractable epilepsy. In order to localize the focus of the seizure so that part of the brain can be resected, these patients are implanted with intercranial electrodes. This clinical necessity provides the unique opportunity to record from single neurons while the subjects participate in behavioral tasks. The behavioral tasks are designed to probe the function of neurons in the anterior cingulate cortex, the region of the brain we study. The anterior cingulate cortex is particularly relevant when studying those characteristics that make the higher primates, particularly humans, adept problem solvers. When making a decision, one must integrate many types of emotional basis do I make my decision? Which aspects of the scenario do I ignore, and which do I attend? EEG recordings show that a theta (5-8 Hz) rhythm with its source in the anterior cingulate is associated with tasks requiring intense concentration. The amplitude of this signal is reduced by the presence of anxiety, and increases with increasing task difficulty. In what may be a related phenomenon, a sharp downward deflection of the EEG signal (error related negativity or ERN) occurs when the subject makes an error during task performance or when her choices may indicate a negative outcome. By studying both the intercranial EEG and the single neuron activity in the implanted patients, we hope to understand how single neuron responses relate to these signals.

One of our behavioral paradigms, the Stroop task, requires that the subject respond to the color of the ink of a written word. The correct answer, for example, "red," can conflict with the meaning of the word itself, for example "green." Such conflicts are known to result in longer reaction times and high error rates, and activate the ACC in fMRI studies. In another paradigm we ask the subject to reply whether a particular adjective describes themselves. An EEG study has shown that a "yes" response to a negative word, for example "bossy," can result in an ERN, as if the person has made an error! In an additional paradigm, we simulate foraging activity by having the subject makes a series of decisions with various gains and losses associated with each decision. Through this paradigm we can study anticipation and acquisition of reward, unexpected reward or loss, "strategy shift" when responding to reduced reward, and investment, where the subject undergoes a series of small losses in order to ultimately gain a large reward.

[1]*Huntington Hospital, Pasadena, CA*

Reference

Bush G., Luu, P. and Posner, M.I. (2000) *Trends Cogn. Sci*. **4**:215-222.

365. Morphological and molecular characterization of the spindle cell in anterior cingulate cortex

Karli Watson, Nora Tu[1], Sarah Teegarden[2], John Allman

Spindle neurons are unique among primates to the great apes and to humans, and they are the only known cellular specialization of its kind. Spindle cells are large in size (long axis 50-120 μm) and distinctive in shape. Unlike the surrounding pyramidal neurons, spindle cells are long and thin, with prominent and symmetrical apical and basal dendrites. They are mostly restricted to the anterior cingulate cortex, where they appear inhomogenously distributed throughout layer Vb. This layer of cortex is an output layer, sending signals that have been processed locally in the cingulate cortex to other regions in the brain. The somas of these cells have volumes that scale with encephalization, with the biggest-brained apes, humans, having the largest spindle cells. This suggests that the spindle cells have far-reaching projections, perhaps to coordinate other regions to the rhythms of the anterior cingulate. What is the function of these cells? Given the late timing of they phylogenetic appearance, do they play some role in mediating the enhanced cognitive capacities that we associate with the great apes and humans? There are technical limitations to studying these unique cells for example, morphological identification of these cells after *in vivo* electrophysiological recording is impossible.

Mainen and Sejnowski showed that firing patterns derived from cells modeled in NEURON varied greatly depending on the morphology of the modeled cell. The unique morphology of the spindle cell might allow us to predict its trademark-firing pattern, which then could possibly allow us to recognize a spindle cell from electrophysiology. In order to determine the dendritic morphology of the spindle cell, we applied the "DiOlistic" technique to fresh brain tissue received the same day from a hemispherectomy operation. This yielded neuroanatomical details of the spindle cells, similar to afluorescent rendition of a Golgi-stain. These results will serve as the basis for predicting a theoretical firing pattern. We are also investigating the chemical components of neurons in the anterior cingulate by immunohistological methods. We have found GAT-1 immunoreactive cartridges in layer V anterior cingulate cortex. These cartridges are the inputs of GABAergic cells lined densely along the proximal part of pyramidal, and possibly spindle, cell axons. We have also found that the dopamine D3 receptor selectively stains the soma and apical dendrites of spindle and layer V pyramidal cells, which may be related to receipt of reward signals mediated by dopamine.

[1]*Biology undergraduate, Caltech*
[2]*SURF student, Caltech*

Reference

Mainen, Z. and T. Sejnowski (1996) *Nature* **382**:363-366.

366. The development of spindle cells in human anterior cingulate cortex

Atiya Hakeem, Nicole Tetreault, John Allman

Our qualitative observations suggest that the spindle cells are not present in newborn human infants and first appear at about four months of age. At this stage, the apical and basal dendrite of the spindle cells are often twisted in corkscrew-like configurations that resemble flagellae. They also occur in vertical tandem arrays with the apical dendrite of one cell contacting the basal dendrite and soma of another. During the period from four months to one year postnatally, the spindle cells are quite abundant and some appear to be ungoing apoptosis. To quantify these observations, we are mapping the distribution of spindle cells in brains of different ages using stereological sampling procedure in the Yakovlev brain collection at the National Museum of Health and Science.

367. Brain activation in auction bidding: A fMRI study

David Grether[1], Daniel Rowe[2], Charles Plott[1], Marty Sereno[3], John Allman

We report the results of experiments in which human subjects in a 1.5T Siemens scanner were given coupons redeemable for cash after the experiment. Subjects could keep the coupons or sell them in auctions, keeping instead the sales price. Each subject participated in a series of thirty-two auctions with the bids to buy the coupons generated randomly prior to the experiment. The subjects' task was to choose prices at which they were willing to sell the coupons. After sixteen auctions subjects were given the strategy that would maximize their revenues from the auction, and then sixteen more auctions were performed. The data were analyzed using a Bayesian factor analysis. We found significant activation in the frontal pole especially in Broadmann's area 10, the anterior cingulate cortex, the amygdala and the basal forebrain. The anterior cingulate cortex and basal forebrain receive heavy dopaminergic input from the midbrain tegmentum, which signals expectation of reward. The amgydala is typically active in fearful or aversive situations, perhaps related to fear of loss in this instance. Brodmann's area 10 has expanded greatly in the evolution of the brain in humans and participates in episodic memory, risk-reward assessment, and moral reasoning. There were systematic differences in brain activation before and after subjects had the solution to the problem. The frontal pole and basal forebrain were significantly more active during the first 16 auctions than in the second set after the solution had been provided. Comparing the eight auctions immediately before and after the solution was revealed, there was a significant drop in the activation of activation of anterior cingulate cortex.

[1]*Economics Professors. Humanities and Social Sciences, Caltech*
[2]*Professor, Medical College, Wisconsin*
[3]*Professor, Cognitive Science, University of California, San Diego, CA*

368. White matter gray matter scaling in the cerebellum

Eliot Bush, John Allman

A number of years ago we noted that as neocortex size increases, the proportion of white matter increases disproportionately. In the neocortex, larger size demands extra investment in wiring. We are now investigating white matter gray matter scaling in another prominent laminar structure: the cerebellum. Using the comparative mammalian histology collection at the University of Wisconsin, we have measured the amount of gray and white matter in a sample of over 40 mammalian species. We find that the relationship in cerebellum differs markedly from that in neocortex. Regression of log white matter volume on log gray matter volume shows that the proportion of white matter increases more slowly in the cerebellum. The cerebellar regression coefficient of 1.13 differs significantly from the neocortical coefficient of 1.28. This likely reflects the fact that, in comparison with neocortical circuitry, cerebellar circuitry is largely feed-forward, and contains far fewer long-range horizontal connections. Such differences effect the wiring adjustment evolution must make as brain size increases.

369. Comparison of white matter gray matter scaling in three regions of the primate neocortex

Eliot Bush, John Allman

We have compared white matter gray matter scaling in three different regions of primate neocortex. In primate histological material stained for cell bodies, one can readily distinguish the primary visual cortex from surrounding regions. Similarly, the border between primary motor cortex and primary somatosensory cortex can also be recognized based only on histology. Taking advantage of these borders, we have divided the neocortex of 20 primate species into three regions. We have then measured white matter and gray matter volumes for the three. Regression of log white matter volume on log gray matter volume for each of the three reveals that their regression coefficients do not differ significantly. However, the ratio of white matter to gray matter is less for primary visual cortex than for the other two. As primary visual cortex size increases, its white matter component increases with the same exponent as for other regions. However, at any given brain size, the ratio of white matter to gray matter is less for primary visual cortex than for the other two regions.

Publications

Allman, J.M. (2001) Evolving Brains. Nikkei Science (Japanese edition).

Allman, J.M., Watson, K. and Hakeem, A. (2002) Two phylogenetic specializations in the human brain. *Neuroscientist* **8**:335-346.

Bush, E.C., Shimojo, S. and Allman, J.M. (2002) Rapid learning in a collinear edge pop-out detection task. *Perception* **31**:791-798.

Bush, E.C., Simons, E.L., Dubowitz, D.J., Scading, M., Nelson Jr., M.J. and Allman, J.M. (2002) Endocranial volume and optic foramen size in Parapithecus grangeri. In: "Anthropoid Origins," C. Ross, ed. In press.

Rosenbluth, D. and Allman, J.M. (2002) The effect of gaze angle and fixation distance on the responses of neurons in V1, V2, and V4. *Neuron* **33**:143-149.

Tetreault, N., Hakeem, A. and Allman, J.M. (2002) Retinal ganglion cell size and distribution in prosimian primates. In: "Anthropoid Origins," C. Ross, ed. In press.

James G. Boswell Professor of Neuroscience: Richard A. Andersen
Visiting Associates: William G. Bradley, Jr., William L. Caton, Igor Fineman, Mel Goodale, Partha Mitra
Member of the Professional Staff: Boris Breznen
Senior Research Fellows: Hans Scherberger
Research Fellows: Hemant Bokil, Chris Buneo, Brian Corneil, David Dubowitz, Bradley Greger, Sam Musallam, Zoltan Nadasdy, Zoran Nenadic[1], Bijan Pesaran, Alexander Polyakov, Rodrigo Quian Quiroga, Elizabeth Torres, Shau-Ming Wu
Graduate Students: Kyle Bernheim, Rajan Bhattacharyya, Eddie Branchaud[1], Lance Cai[1], Michael Campos, Shiyan Cao[1], Brian Lee, Daniella Meeker, Dylan Nieman
Research and Laboratory Staff: Mike Andersen, Kristen Andersen, Kevin Duraj, Betty Gillikin, Cierina Marks, Lori Rose, Viktor Shcherbatyuk, Kelsie Weaver
[1]*Mechanical Engineering, Caltech*

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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.

Recently we have begun a project attempting to develop a neural prosthesis for paralyzed patients. This prosthetic system will be designed to record the electrical activity of nerve cells in the posterior parietal cortex of paralyzed patients, interpret the patient's 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 or a computer to surf the internet. Currently we are testing whether intentional signals can be deciphered by recording the activity of single cells in the reach region of healthy monkeys. We have found that we can predict where an animal plans to reach in a task in which he is instructed to plan to reach in one of two directions. We provided feedback to the animal regarding this prediction, and the animal quickly learned that he could receive his juice reward at the end of each trial by simply thinking about reaching in the correct direction without actually reaching. We are currently expanding this research to record from multiple cells using arrays of implanted electrodes. This approach will allow us to increase the number of potential reach directions that can be decoded. If these multicellar recording experiments are successful, in principle this approach could be used to record from the posterior parietal cortex of patients with paralysis, enabling them to operate external devices with their movement thoughts.

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 in primates.

Another major effort of our lab is to examine the neural basis of motion perception. One series of experiments is determining how optic flow signals and efference copy signals regarding eye movements are combined in order to perceive the direction of heading during self-motion. These experiments are helping us understand how we navigate as we move through the world. A second line of investigation asks how motion information is used to construct the three-dimensional shape of objects. We asked monkeys to tell us which way they perceived an ambiguous object rotating. We found an area of the brain where the neural activity changed according to what the monkey perceived, even though he was always seeing the same stimulus. In other experiments we have been examining how we rotate mental images of objects in our minds, so-called mental rotation. In the posterior parietal cortex we find that these rotations are made in a retinal coordinate frame, and not an object based coordinate frame, and the mental image of the object rotates through this retinotopic map.

We have successfully recorded functional magnetic resonance signals following visual activation of the monkey brain. This finding 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.

370. Rapid plasticity in the parietal reach region demonstrated with a brain-computer interface

Daniella Meeker, Shiyan C. Cao, Joel W. Burdick, Richard A. Andersen

It has become increasingly evident that parietal cortex plays an important role in sensory-motor transforms, and may be important for the type of adaptation necessary for realignment of sensory-motor maps. Cells in the parietal reach region (PRR) of the posterior parietal cortex of macaque monkeys encode the plans to make reach movements. We trained monkeys to use their intentions, decoded from PRR neurons in real-time with a probabilistic algorithm, to position a cursor on a computer monitor without actually making a reach movement. The monkeys rapidly acquired the task for all significantly tuned cells recorded during a delayed reach paradigm. Approximately half of the cells recorded from two monkeys showed a rapid adaptation to the cursor positioning task, by increasing the firing rate when the cursor target was presented in the preferred direction and/or decreasing the firing rate in the null direction within several trials. Control experiments show that the PRR activity does not predict the direction of intended eye movements or the direction of attention. The finding that the animals can quickly abstract their intended reach signals for cursor control, and rapidly learn to change this activity to improve performance in the task, suggests that PRR signals could be used to control neural prosthetic systems for paralyzed patients.

371. Unsupervised spike detection and sorting using wavelets

Rodrigo Quian Quiroga, Zoltan Nadasdy, Yoram Ben-Shaul, Bijan Pesaran, Anatol Bragin, Richard A. Andersen

Increasing advances in acquisition systems allow the recording of several channels simultaneously. However, these advances have not been followed-up by advances in the processing of the data. In fact, manual spike sorting of as many as e.g. 64 channels seems a very time consuming task and is nearly impossible to perform during an experiment. We present a fast method to automatically detect and sort spikes from multi-unit recordings. The method is based on the wavelet transform, which is a time-frequency decomposition of a signal with optimal resolution both in the time and in the frequency domains. In the first step, spikes are detected as deviations in the pattern of the time-frequency decomposition. This approach, instead of an amplitude threshold, allows the detection of small spikes embedded in the background noise. In the second step, a small set of wavelet coefficients from each spike is chosen as input to a clustering algorithm. The advantage of using wavelets is that, with only a few coefficients, we can recognize time-localized differences in the shape of the spikes corresponding to different neurons. Finally we use a 'Super-Paramagnetic Clustering' (Blatt *et al*., 1996) algorithm that allows an automatic separation of clusters and successfully deals with clusters without well-defined centers and/or large variances. By choosing appropriate criteria, this whole process of detection and sorting is performed without supervision. Moreover, due to its speed, the algorithm is suitable for on-line analysis.

372. Comparison of unsupervised algorithms for on-line and off-line spike sorting

Zoltan Nadasdy, Rodrigo Quian Quiroga, Yoram Ben-Shaul, Bijan Pesaran, Daniel A. Wagenaar, Richard A. Andersen

The goal of spike sorting is to identify the extracellularly recorded multiunit activity with discrete neuronal sources. To perform this task, spike-sorting algorithms consist of three independent steps: spike detection, spike projection and clustering. Attempts to accelerate these steps by using unsupervised algorithms have been made but the success of such methods is highly dependent on assumptions related to the statistics of signals relative to the noise component that is specific for the given brain area and recording technique. We present a new method that: (1) Does not assume spike shapes to follow any specific distribution; (2) is unsupervised; and, (3) can be applied during the data collection.

First, we compare different methods of spike detection (threshold, slope, energy, template and wavelet) using the signal detection theory on simulated extracellular multiunit recordings. Second, we introduce a new method using "circular embedding" to project spike shape differences to a multidimensional space. Third, we compare the separability of such projections with that of the principal components and wavelet coefficients. Fourth, we employ "superparamagnetic clustering" (Blatt *et al*., 1996) and compare the results with K-means, and Bayesian clustering methods. Fifth, we test whether the combined method of "circular embedding" and "superparamagnetic clustering" on multi-dimensional projections is suitable to perform "on-line" during the data acquisition.

373. What do the local field potentials measure

Alexander Polyakov, Richard A. Andersen, Partha P. Mitra

It is relatively straightforward to measure and interpret trains of action potentials since only the times of the spikes matter. Local field potentials (LFP), i.e., the low frequency voltages measured with extracellular electrodes, present further problems. Here, the physical properties of the electrodes, the electrical properties of the surrounding tissue and the microanatomy influence the measurements. This has impeded the interpretation of LFPs. A related problem is that of current source density (CSD) analysis. Here we formulate an inverse problem relating the CSD and the LFP. We compute the associated linear kernel for several electrode geometries, and apply a newly developed methodology for solving linear inverse problems, to develop insight into LFP measurements. Simulations, and data examples of recordings from the parietal cortex of the macaque are used to illustrate the ideas.

374. Decoding temporal structure in local field potential measurements

Hemant S. Bokil, Bijan Pesaran, Richard A. Andersen, Partha P. Mitra

In previous work we presented a general paradigm to the problem of decoding temporal structure in neural data, both spike trains and local field potential (LFP) measurements. Here we present an extensive analysis of LFP recordings made from the lateral intra-parietal area (LIP) and the frontal eye fields (FEF) during a delayed saccade task in awake behaving macaque. For area LIP, we find that over 90% of the trials to the preferred direction, and over 60% of the trials to the neighboring directions are predicted correctly. The beginning of these trials is predicted with a timing accuracy of less 50 ms. We also find that the performance of the method is independent of the presence or absence of spikes in the recordings and that the method allows generalizations between distinct data sets. For FEF, we find that LFP activity segregates into different frequency bands than LIP, but contains similar information about the intended saccade. Finally, we show the potential application of this method to prosthetics by implementing it in real-time.

375. Activity of SEF neurons in an object-based saccade task

Boris Breznen, Richard A. Andersen

We recorded from single neurons in the supplementary eye field (SEF) of a macaque monkey during two types of oculomotor tasks. First, the full 2D movement field of a neuron was mapped using the standard memory saccade task. Next, we used an object-based saccade task where the target was located on one of the two symmetric vertices of an isosceles triangle (OBJ task). After cueing one side of the triangle, the triangle was extinguished and, after a delay, re-displayed at a random location in one of two possible orientations (+/-45°). We examined SEF neurons that showed significant saccadic modulation in the memory saccade task (N=14). All of the selected cells maintained significant saccadic modulation in the OBJ task. We used two-way anova to test three additional sources of variability: Spatial location of the target in retinal coordinates, object-centered location of the target and orientation of the object on the screen. Six of the 14 cells showed modulation due to the spatial location of the target (43%). One cell showed modulation due to the object-centered location of the target and one cell showed modulation due to the object orientation. The data suggest that the neurons in SEF are mostly responsive to the retinal location of the target.

376. Inverse BOLD fMRI phasing in V1 during a visual motion task

Kyle A. Bernheim, David J. Dubowitz, Richard A. Andersen

Blood oxygen level dependent (BOLD) methods of functional magnetic resonance imaging (fMRI) rely upon dynamic vascular changes during a given task. These changes demonstrate an upregulation in blood supply to cortical areas relative to their level of activation. This upregulation is evident as an fMRI signal increase positively correlated to the stimulus timecourse, however, inverse correlation has also been reported.

We have found evidence of bilateral functional deactivation in human V1 during a visual motion stimulus. Subjects fixated at the center of a series of concentric rings of alternating black/white contrast spanning 22° of the visual field. An imaging trial was divided into epochs during which the right or left visual hemifield exhibited smooth expansion or contraction while the opposing field was stationary. The visual field then went briefly dark about the fixation point, and was followed immediately by a static field of concentric rings.

Portions of V1 near the occipital pole (likely corresponding to foveal receptive fields) demonstrated the expected result of increased signal upon stimulation. Conversely, functional volumes in and about the anterior reaches of the calcarine sulcus exhibited an increase in fMRI signal positively correlated to the offset of stimulation. These volumes were present bilaterally and well separated into each cerebral hemisphere. Signal suppression from baseline during bright epochs indicates a relative decrease in blood supply to these extrafoveal areas. This functional deactivation may serve to shut down areas of cortex in favor of areas relevant to the current visual task.

377. Spike triggered averaging of natural scanning reveals the dynamics of multiplexed eye and head signals in the primate superior colliculus

Michael Campos, Anil Cherian, Mark A. Segraves

In previous reports we described the existence of eye and head centered activity exhibited by superior colliculus neurons during a natural scanning task (Campos *et al.,* 2000; Cherian *et al.,* 2001). Here we report on the dynamics of the switching between the head-centered encoding of eye position and the eye -centered encoding of saccade vector. We defined a spike triggered event as eye positions and saccade vector metrics associated with action potentials that occurred within a time window defined in relation to the onset of a saccade. These events were tested with the Rayleigh test for circular data to determine if the triggered averages are significantly tuned. In our analysis of 72 intermediate layer SC neurons from two macaques, we found 68 of 72 ($P<.001$) to have significant tuning for saccade vector at the time of saccade onset, as expected. In contrast, 150+/- 10 ms before the saccade, neurons were not as well tuned for saccade vector, but showed significant tuning for the current or intended eye position. The spike triggered average transitioned into being predictive of the upcoming saccade vector between 100 and 60 ms before the saccade, when the tuning of the intended eye position dropped. Thus, the encoding of the position of the eye in the head is less temporally precise as the instruction to move the eyes, which dominates the activity of the SC around the time of a saccade. We suggest that the multiplexed nature of the signals sharing the same neural substrate could facilitate the coordination of eye and head movements in gaze shifts.

378. Effects of gaze angle and vision of the hand on reach-related activity in the posterior parietal cortex

Chris A. Buneo, Richard A. Andersen

We are currently exploring the effects of gaze angle and vision of the starting position of the hand on the responses of reach-related neurons in the posterior parietal cortex (PPC). Single cell recordings were obtained from one monkey during the performance of an instructed-delay reaching task in which initial hand position in eye coordinates (H) and target position in eye coordinates (T) were varied at two fixed gaze angles. For each gaze angle, we quantified the relative importance of H, T and H-T (i.e., the movement vector) in modulating cell activity by determining the axis within (H, T) space that corresponded to the greatest change in firing rate. In 86% (N=14) of the neurons tested, the orientation of this axis varied with gaze angle ($p<0.05$). This variation in the relative coding of reach-related variables may reflect the influence of body-centered information on cell responses or systematic differences in the reach trajectories associated with different workspaces. In a subset of cells (N=7), the effect of vision of the starting position of the hand was examined by interleaving sets of trials in which vision of the starting position was alternately present ('vision' condition) or absent ('no vision' condition). For all of the neurons tested, the relative importance of H, T and H-T varied for the 'vision' and 'no-vision' conditions ($p<0.05$). This variation suggests that the representation of reach-related variables at the single cell level in the PPC depends in part on visual information regarding the starting position of the hand.

379. Multi-jointed arm movement: A model

Elizabeth Torres

Goal-directed arm motion paths arise as a function of the task at hand. Different tasks emphasize different goals. For example, introducing an obstacle in a reach-to-grasp action changes the resulting paths due to a shift in goal priority. In optimization, goals can be expressed in terms of cost functions. We present a geometric model that capitalizes on this fact. The model uses a gradient technique that relies on the pulled-back metric from hand space into the space of postures to resolve endpoint path selection, posture-change specification, error correction and multiple constraint satisfaction on line without pre-planning. The resulting paths in posture space are locally geodesics of the cost surface (shortest distance paths with respect to the curvature of the cost surface). The advantage of this approach is that simple quadratic functions linked to the cost can be used to encode the space of solutions corresponding to a given task. The model is instantiated in an arm with seven degrees of freedom that has to position and orient the hand in space. General predictions are: (1) No effect of speed on the postural paths of the arm; (2) the initial arm posture and the target orientation affect the motion paths both in posture and in endpoint space; (3) the transport and orientation errors in the movement decrease proportionally regardless of the above manipulations in movement parameters. Simulated orientation-matching movements are compared to actual human movement data to assess the validity of several of the model's behavioral predictions.

380. Variable kernels in signal processing of spikes leads to complex neuron behavior

Sam Musallam, Robert D. Tomlinson

Much research is currently aimed at elucidating the information that spike trains convey. However, the real observer of the spike train, the post synaptic neuron, responds to the information contained within the presynaptic spike train through some function of synaptic and membrane dynamics. Here, we model the current in the post synaptic neuron in response to pre-synaptic spikes as: $K(t)=pe^{-t/T_s}+g_f e^{-t/T_f}$ where T_f refers to the fast time constant (e.g., AMPA receptors), T_s to the slow time constant (e.g., NMDA receptors), ρ and g_f are the proportion of channels open. Neuronal behavior was modeled by allowing the gain ρ to vary as a function of the input-firing rate. Our simulations demonstrate that such dynamics can explain several types of neural computations such as: 1) Asymmetric integration in the vestibular nucleus; 2) increase in the bias of a post-synaptic vestibular neuron in response to an increase in the stimulus input; and, 3) gain fields, as observed in the parietal cortex. Simulations will also be presented that show that this gain manipulation can be modeled as changes in the time constants T_s, leading to the generation of an adaptive filter. Our modeling efforts demonstrate that complex behavior can arise from the properties of a single synapse without resorting to any kind of neural system or complicated neural network. Such a simple approach may explain many observed nonlinear properties in neural systems.

381. Gain adaptation does not transfer between visually-guided and memory-guided saccades under conditions of dim illumination

Bradley Greger, Richard A. Andersen

A previous study of saccadic adaptation in monkeys showed on average an 88% transfer of adaptation from visually-guided to memory-guided saccades, indicating a common site of neural plasticity (Fuchs, 1996). A study of saccadic adaptation in humans showed that there was little or no transfer of adaptation between visually-guided and memory-guided saccades, suggesting independent sites of neural plasticity (Deubel, 1995). We trained a monkey to make visually-guided and memory-guided saccades using a digital light projection system. Saccades were made from a central fixation point to eccentric targets randomly placed between 7.5-15° in one of several directions. Both visually-guided and memory-guided saccades were gain adapted by back-stepping the location of each target by 30% during the saccade. The gain of both visually-guided and memory-guided saccades was measured before and after the adaptation of either saccade type. A t-test was used to check for significant changes in gain for both the adapted and non-adapted saccade type. We show that the adaptation of visually-guided saccades did not result in a significant change in the gain of memory-guided saccades or vice versa. Adaptation was not transferred, suggesting distinct sites of neural plasticity. It is possible that the dim illumination

present in Duebel's and our studies allowed the gain adaptation of visually-guided and memory-guided saccades to take place using different spatial representations and therefore at different sites of neural plasticity, while the lack of illumination in Fuchs' study enforced a single spatial representations and a common site of neural plasticity.

Publications

Andersen, R.A. and Buneo, C.A. (2002) Intentional maps in posterior parietal cortex. *Ann. Rev. Neurosci.* **25**:189-220.

Andersen, R.A. and Buneo, C.A. (2002) Sensory-motor integration in the posterior parietal cortex: Specific, intermediate, and network properties. In: "The Parietal Lobes," A. Segal, R.A. Andersen, H.O. Freund, and D.D. Spencer, eds., Lippincott, Williams and Wilkins, Philadelphia, PA. In press.

Batista, A.P. and Andersen, R.A. (2001) The parietal reach region codes the next planned movement in a sequential reach task. *J. Neurophysiol.* **85**:539-544.

Buneo, C.A., Jarvis, M.R., Batista, A.P. and Andersen, R.A. (2002) Direct visuomotor transformations for reaching. *Nature* **416**:632-636.

Cohen, Y.E. and Andersen, R.A. (2002) A common reference frame for movement plans in the posterior parietal cortex. *Nature Rev. Neurosci.* **3**:553-562.

Cohen, Y.E. and Andersen, R.A. (2002) Multimodal representations of space in the posterior parietal cortex. In: "Handbook of Multisensory Integration," G. Calvert, C. Spence, and B. Stein, eds. MIT Press, Cambridge, MA. In press.

Cohen, Y.E. and Andersen, R.A. (2002) Multimodal spatial representations in the primate parietal lobe. In: "Crossmodal Space and Crossmodal Attention," J. Driver and C. Spence, eds., Oxford University Press.

Cohen, Y.E., Batista, A.P. and Andersen R.A. (2002) Comparison of neural activity preceding reaches to auditory and visual stimuli in the parietal reach region. *NeuroReport* **13**:891-894.

Crowell, J.A. and Andersen R.A. (2001) Pursuit compensation during self-motion. *Perception* **30**:1465-1488.

Dubowitz, D.J., Bernheim, K.A., Chen, D-Y., Bradley, W.G. and Andersen, R.A. (2001) Enhancing fMRI contrast in awake behaving primates using intravascular magnetite dextran nanoparticles. *Neuroreport* **12**:2335-2340.

Dubowitz, D.J., Chen, D.-Y., Atkinson, D.J., Scadeng, M., Martinez, A., Andersen, M.B., Andersen, R.A. and Bradley Jr., W.G. (2001) Direct comparison of visual cortex activation in human and non-human primates using functional magnetic resonance imaging. *J. Neurosci. Methods* **107**:71-80.

Grunewald, A., Bradley, D.C. and Andersen, R.A. (2002) Neural correlates of structure-from-motion perception in macaque V1 and MT. *J. Neurosci.* **22**:6195-6207.

Li, C.-S. and Andersen, R.A. (2001) Inactivation of macaque area LIP delays initiation of the second saccade from contralesional eye positions in a double-saccade task. *Exp. Brain Res.* **137**:45-57.

Mojarradi, M., Binkley, D., Blalock, B., Andersen, R.A., Ulshoefer, N., Johnson, T. and Del Castillo, L. (2002) A miniaturized, neuro-prosthesis suitable for implantation into the brain. *IEEE Trans. Neural Systems and Rehab. Eng.* In press.

Pesaran, B., Pezaris, J., Sahani, M., Mitra, P.M. and Andersen, R.A. (2002) Temporal structure in neuronal activity during working memory in macaque parietal cortex. *Nature Neurosci.* **5**:805-811.

Sabes, P.N., Breznen, B. and Andersen, R.A. (2002) The parietal representation of object-based saccades. *J. Neurophysiol.* In press.

Scherberger, H. and Andersen, R.A. (2002) Sensorimotor transformation in the posterior parietal cortex. *The Visual Neurosciences*, MIT Press. In press.

Shenoy, K.V., Crowell, J.A. and Andersen, R.A. (2002) Pursuit speed compensation in cortical area MSTd. *J. Neurophys.* In press.

Sugihara, H., Murakami, I., Shenoy, K.V. and Andersen, R.A. (2002) Responses of MSTd neurons to simulated 3D-orientation of rotating planes. *J. Neurophysiol*. **87**:273-285.

The Lois and Victor Troendle Professor of Cognitive and Behavioral Biology and Professor of Computational and Neural Systems: Christof Koch
Visiting Professors: Joseph Bogen, Heinz Schuster[5]
Research Fellows: Kamran Diba, Fred Hamker, C.J. Han, Whee Ky Ma, Colm O'Tuathaigh, Rodrigo Quian Quiroga, Christoph Rasche, Rufin Van Rullen, Patrick Wilken
Graduate Students: Ronald McKell Carter, Grace Chang[1], Carl Gold[1], Constanze Hofstötter[1], Alex Holub[1], Gabriel Kreiman, Ania Mitros[1], Chunhui Mo[2], Farshad Moradi[1], Shreesh Mysore[3], Alberto Pesavento[4], Robert Peters[1], Leila Reddy[1], Naotsugu Tsuchiya[1], Dirk Walther[1]
Research and Laboratory Staff: David Kewley, Mark Ryback, Candace Vavra
[1]*Computation and Neural Systems graduate student, Division of Engineering and Applied Science*
[2]*Biochemistry graduate student, Division of Chemistry and Chemical Engineering*
[3]*Control and Dynamical Systems graduate student, Division of Engineering and Applied Science*
[4]*Electrical Engineering graduate student, Division of Engineering and Applied Science*
[5]*University of Kiel, Germany*

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Summary: Research in the laboratory of Professor Christof Koch focuses on three areas: (1) biophysics of computation in single neurons; (2) understanding visual selective attention and visual consciousness at the neuronal, behavioral and computational levels; and (3) studying the role of attention, working memory and awareness in associative fear conditioning in humans and mice. For more details and all publications, see http://www.klab.caltech.edu.

Research carried out in our group as part of a program called "Biophysics of Computation" studies how the biophysics, synaptic architecture and dendritic architecture of individual neurons subserve information processing. This research has been summarized in a textbook, "Biophysics of Computation: Information Processing in Single Neurons," by C. Koch and published by Oxford University Press in 1999. To what extent do neuronal noise sources (thermal noise, channel noise, noise due to synaptic background firing, and so on) limit signal detection or signal reconstruction at the level of individual neurons? What are the biophysical mechanisms underlying neuronal computations? How do neurons multiply? How complex are single nerve cells? What is the code used to transmit this information? Analytical work, backed up by detailed computer simulations of nerve cells based on electrophysiological data from our experimental collaborators at the Hebrew University in Jerusalem (Idan Segev and Yossi Yarom) and elsewhere, is used to generate experimentally verifiable predictions. We are also analyzing the variability, reliability, and randomness of multi-unit firing activity from cells in medial temporal and frontal lobes in human patients, different stages in the electrosensory system of the weakly electric fish *Eigenmannia*, as well as in simulated networks in order to understand the existence and precision of the temporal code used by nerve cells to transmit information.

Understanding complex information-processing tasks, in particular the action of selective, visual attention (both saliency-driven bottom-up as well as task-dependent, top-down form) requires a firm grasp of how the problems can be solved at the "computational" level, and how the resulting algorithms can be implemented onto the known architecture of the visual cortex and associated subcortical areas. We use analytical methods, coupled with detailed computer simulations of the appropriate circuitry in the primate visual system, to study how these neuronal networks control selective visual attention and how they give rise to motion perception, object discrimination and detection (in collaboration with Tomasio Poggio at MIT and Laurent Itti at USC). The resulting algorithms are being applied to problem in image analysis and machine vision. Researchers in our laboratory study visual perception in the presence and (near)-absence of selective visual attention as well as our ability to classify and distinguish two-dimensional visual patterns using psychophysical techniques in normal subjects. We are complementing some of these studies using noninvasive fMRI imaging under the identical stimulus protocols. Our laboratory continues to collaborate with Itzhak Fried at UCLA in recording single units from multiple electrodes in the medial temporal cortex of awake patients during visual perception, rivalry, and imagery.

We continue to collaborate with Francis Crick (Salk) to develop a neuronally-based framework to understand how subjective feelings (e.g., as in conscious visual perception) can arise in the mammalian forebrain. In order to make experimentally verifiable progress on the mind-brain problem, it will be critical to interfere deliberately, transiently, rapidly and reversibly with groups of genetically identifiable forebrain neurons in experimental animals. In collaboration with David Anderson (Caltech), Henry Lester (Caltech) and Michael Fanselow (UCLA), we have developed an associative fear conditioning paradigm in both mice and normal human

subjects. We are investigating the role of working memory, attention and awareness in acquiring delay versus trace conditioning in both species and in identifying their underlying neuronal representations.

382. Modeling and analysis of extra-cellular recording probabilities

Carl Gold[1]

We use simulations and analysis of biophysics to investigate the probability of detecting different neuron types with extra-cellular electrode recordings. Simulations are carried out in the NEURON simulation environment and the time course of the electric field around the neuron is calculated. The field strength is analyzed along with the typical noise present in extra-cellular recordings to estimate the distance from which an electrode could detect a particular cell type and the probability of detection for the neuron concentrations in different brain regions. The research is in progress and will include an analysis of the effect of neuron size, shape and channel density on the difficulty of detecting different neurons and an analysis of the consequences for recording in different areas of the brain. The goal is to quantify the well-known but poorly studied issue of "big neuron" bias in electrophysiology.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*

383. Subthreshold voltage fluctuations in pyramidal neurons

Kamran Diba, Gilad Jacobsen[1]*, Yasmin Oz*[1]*, Christof Koch, Idan Segev*[1]*, Yosef Yarom*[1]

By combining a theoretical and an experimental approach, we investigate the power spectral density of sub-threshold voltage noise in the cell body of neocortical pyramidal cells from layer 4/5 rat brains. Subthreshold voltage noise can affect the temporal precision of a neuron's action potentials and their I-F (input current vs. firing frequency) relationship, and has also been suggested to play a role in the detection of weak signals ("stochastic resonance"). Theoretically, we calculate the contributions of stochastic ion channels distributed in a ball and stick model of the cell by applying the theory of Markov chains to ion channel kinetic schemes. We also account for the contributions of thermal agitations and random synaptic release. Experimentally, the voltage power spectrum is measured at different holding potentials under current clamp mode. The cells are hyperpolarized to enable calculation of the power spectral densities without interference from spontaneous action potentials. It is observed that the noise variance increases with depolarization, as expected by theoretical calculations. The addition of pharmacological agents, including TTX, TEA, DNQX, and bicuculline, further enable us to gauge the relative contributions of different sources of noise. However, we observe the rather puzzling behavior that the spectrum of voltage fluctuations above 10 Hz does not depend on the holding potential of the cell.

[1]*Institute of Life Sciences, Hebrew University, Jerusalem, Israel*

384. Unsupervised spike sorting with wavelets and superparamagnetic clustering

Rodrigo Quian Quiroga, Zoltan Nadasdy, Yoram Ben-Shaul[1]

We describe a new method for detecting and sorting spikes from multiunit recordings. We will show several implementation criteria that make the algorithm completely unsupervised and fast, therefore being suitable for on-line implementations. The two cornerstones of the method are: 1) selection of spikes features by using the wavelet transform; and, 2) use of superparamagnetic clustering. By using wavelet coefficients as feature detectors we are able to separate small and localized differences in the spike shapes of the different units. Moreover, the use of superparamagnetic clustering allows an automatic clustering of the data without constraints such as a well-defined mean of the clusters, low variance, Gaussianity, etc.

We quantify the outcome of the algorithm in simulated data closely resembling the conditions of real recordings. For the four examples analyzed the algorithm had a very good performance, with only a few numbers of misclassifications.

[1]*ICNC, The Hebrew University, Jerusalem, Israel*

385. A synaptic learning rule for local synaptic interactions between excitation and shunting inhibition

Chun-Hui Mo[1]*, Christof Koch*

The basic requirement for direction selectivity is a non-linear interaction between two different inputs in space-time. In some models, the interaction occurs at the dendritic-tree level among excitation and inhibition of the shunting type. How is the required spatial specificity achieved using an unsupervised learning rule? We here propose an inhibition-directed, activity-based, unsupervised learning model that may account for direction selectivity in V1 cells. We carried out biophysical simulations in the program NEURON. Our results suggested model cells implementing our learning algorithm developed direction selectivity organically after unsupervised training. Initial connection bias can reduce the training time but is not strictly necessary. We further added a "matching rule" to our learning model. Model cells implementing this additional rule developed direction selective subunit structures on their dendrites. The same learning mechanism could also account for an OFF-ON-ON synaptic connection scheme we reported earlier that was direction selective to both normal and reverse-phi motion. We believe that these learning rules are also applicable—with minor modifications—to retinal direction selectivity.

[1]*Biochemistry graduate student, California Institute of Technology*

386. Dissecting human visual motion system: fMRI and psychophysics

Naotsugu Tsuchiya[1], Christof Koch, Geraint Rees[2], Jochen Braun[3]

We report separate modulation of fMRI activity in primary visual cortex, V1, and in motion sensitive extrastriate cortex, V5/MT+, by changing contrast and coherence of a novel motion stimulus, a multiple wavelets composite. Our finding confirms and unites physiological properties of most extensively studied visual cortical areas, V1 and V5/MT+, found in independent fMRI studies with different stimuli; namely, population activity in V1 correlates with contrast of sinusoidal grating and that in V5/MT+ with coherence of random dot motion. The multiple wavelet composite stimulus would allow us to mimic 'a virtual cortical lesion' invasively and dynamically – turning on and off V1 and V5/MT+ separately – for further study of visual motion.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*
[2]*Institute of Cognitive Neuroscience, University College London, UK*
[3]*Centre for Neural and Adaptive Systems, University of Plymouth, UK*

387. Invariance of object representation in the temporal lobe: Grandmother neurons?

Gabriel Kreiman, Itzhak Fried[1], C. Koch

The controversial idea of grandmother neurons suggests that single cells may encode complex information and that the representation may be invariant to several changes in the stimulus attributes. The question of whether such a representation is actually used by the brain has proven to be difficult to address empirically in monkey single-neuron recordings. Our previous investigations suggest that the human medial temporal lobe encodes visual information using a sparse representation. Preliminary observations have suggested that a single neuron may respond in a highly selective way to strikingly different pictures representing the same person. We are currently investigating whether individual neurons can respond selectively yet showing high degrees of invariance to faces. We are using photographs, drawings and caricatures of famous people as well as photographs of the subject's relatives. Subjects are patients with pharmacologically intractable epilepsy who are implanted with depth electrodes in order to localize the seizure focus for potential surgical resection. We record the activity of multiple individual neurons in the entorhinal cortex, amygdala, hippocampus and parahippocampal gyrus while subjects perform a simple identification task and report whether they can recognize the face images.

[1]*Division of Neurosurgery, UCLA School of Medicine, Los Angeles, CA*

388. Single-neuron correlates of subjective vision in the human medial temporal lobe

Gabriel Kreiman[1], Itzhak Fried[2], Christof Koch

Visual information from the environment is transformed into perceptual sensations through several stages of neuronal processing. Flash suppression constitutes a striking example where the same retinal input can give rise to two different conscious visual percepts. We directly recorded the responses of individual neurons during flash suppression in the human amygdala, entorhinal cortex, hippocampus and parahippocampal gyrus, allowing us to explore for the first time the neuronal responses in untrained subjects at a high spatial and temporal resolution in the medial temporal lobe. Subjects were patients with pharmacologically intractable epilepsy implanted with depth electrodes to localize the seizure onset focus. We observed that the activity of two-thirds of all visually selective neurons followed the perceptual alternations rather than the retinal input. None of the selective neurons responded to a perceptually suppressed stimulus. Therefore, the activity of most individual neurons in the medial temporal lobe of nave human subjects directly correlates with the phenomenal visual experience.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*
[2]*Division of Neurosurgery, UCLA School of Medicine, Los Angeles, CA*

389. Structural description with classified polygon and surface features and their groupings

Christoph Rasche, Christof Koch

We have developed a recognition system that categorizes furniture objects depicted in line drawings. In a feature extraction process, polygons and rectangular surfaces are gradually evolved, classified according to their shape and orientation in 3D space, and grouped according to common grouping regularities. The category representations are expressed by only a few relations of these surface and polygon features and their groupings, and are structurally only loosely formulated in order to be able to cope with the structural variability across different category instances. We use a matching approach to determine the corresponding category representation.

390. Modeling of attentional modulation effects in object recognition

Dirk Walther[1], Max Riesenhuber[2], Tomaso Poggio[2], Laurent Itti[3], Christof Koch

We develop an integrated model for the dorsal (where) and the ventral (what) pathway in the primate's visual processing system and the interaction between these two pathways. To reach our goal of modeling visual search behavior in primates, we integrate and extend the saliency-based model for bottom-up attention by Itti and Koch (2001) and the HMAX hierarchical model for object recognition by Riesenhuber and Poggio (1999a). In the combined model we use saliency-based attention to modulate object recognition at the V4 level. Interesting

regions in the visual scene are successively selected by a rapidly shiftable focus of attention (FOA). Neural activity of a particular neuron in V4 is inhibited based on its distance from the current FOA. Recognition rates for stimuli composed of two paper clip objects typically increase twofold compared to previous experiments without attention (Riesenhuber and Poggio, 1999b). To achieve this improvement a depression of the V4 activity outside the focus of attention by as little as 20% proves to be sufficient. With 10% activity modulation recognition still improves by 70%. We find that the twofold increase in recognition rate is robust over a large range of modulation strengths of the V4 activity. We conclude that a rather weak attentional modulation of the neural activity at the V4 level suffices to recognize multiple objects in the same display. Our model will be extended to search for specific objects in cluttered natural scenes an include biasing of the attention system in a top-down manner. This work was presented at the Annual Meeting of the Cognitive Neuroscience Society in San Francisco, April 14-16 2002 (Walther *et al.*, 2002).

[1]*Computation and Neural Systems graduate student, California Institute of Technology*
[2]*Center for Biological and Computational Learning, Massachusetts Institute of Technology, Cambridge, MA*
[3]*Dept. of Computer Science, University of Southern California, Los Angeles, CA*

References

Itti, L. and Koch, C. (2001) *Nat. Rev. Neurosci.* **2**:194-203.

Riesenhuber, M. and Poggio, T. (1999a) *Nat. Neurosci.* **2**:1019-1025.

Riesenhuber, M. and Poggio, T. (1999b) *Neuron* **24**:87-93, 111-125.

Walther, D., Riesenhuber, M., Poggio, T., Itti, L. and Koch, C. (2002) *J. Cogn. Neurosci.* B14 Suppl. S:46-47.

391. Eye movements are influenced by short-range interactions among orientation channels

Robert J. Peters[1], Laurent Itti[2], Christof Koch

Recent research (Parkhurst *et al*., 2002) showed that a model of bottom-up visual attention can account in part for the patterns of eye movements made by human observers while free-viewing complex natural and artificial scenes. Using a similar method, we tested an enhanced model with excitatory and inhibitory interactions among units at overlapping locations, tuned to different spatial scales and orientations, as inferred from previous psychophysical experiments involving fine discrimination of gabor-like patches in the periphery (Lee *et al.*, 1999). Subject free-viewed images (visual angle 25x20 degrees) from three databases (outdoor photos, fractals, and overhead satellite photos) for 3000 ms per image. An infrared eyetracking system (ISCAN, Inc.) recorded eye position at 120 Hz with a spatial precision of ~0.5 degrees. For each image, we computed the mean model-predicted salience of the points traversed by each subject's scanpath, and judged these values by their z-score in a distribution obtained from random scanpaths of similar length. Across all conditions, the z-scores ranged from 4-14, confirming that in general, our model of bottom-up attention predicts human eye movements with high statistical significance. Moreover, the addition of interactions among oriented units with overlapping receptive fields led to a robust increase in the z-scores, both overall and for individual subjects and image databases. Thus, these interactions, originally modeled after simple gabor-like stimuli viewed under covert attention, also appear to contribute to subjects' overt eye movements under more natural free-viewing conditions.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*
[2]*Dept. of Computer Science, University of Southern California, Los Angeles, CA*

References

Parkhurst, D., Law, K. and Niebur, E. (2002) *Vis. Res.* **42**:107-123.

Lee, D.K., Itti, L., Koch, C. and Braun, J. (1999) *Nat. Neurosci.***2**:375-381.

392. Visual selective behavior can be triggered by a feed-forward process

Rufin VanRullen, Christof Koch

The ventral visual pathway implements object recognition and categorization in a hierarchy of processing areas with neuronal selectivities of increasing complexity. The presence of massive feedback connections within this hierarchy raises the possibility that normal visual processing relies on the use of computational loops. It is not known, however, whether object recognition can be performed at all without such loops, i.e., in a purely feed-forward mode. By analyzing the time course of reaction times in a masked natural scene categorization paradigm, we show that the human visual system can generate selective motor responses on the basis of a single feed-forward pass. We confirm these results using a more constrained letter discrimination task, in which the rapid succession of a target and mask is actually perceived as a distractor. We show that a masked stimulus presented for only 26 ms—and often not consciously perceived—can fully determine the earliest selective motor responses: the neural representations of the stimulus and mask are thus kept separated during a short period of time corresponding to the feed-forward "sweep." Therefore, feed back loops do not appear to be "mandatory" for visual processing. Rather, we found that such loops allow the masked stimulus to reverberate in the visual system and affect behavior for nearly 150 ms after the feed-forward sweep.

393. Detection of objects in natural scenes with minimal or no attention

Fei Fei Li[1], Rufin VanRullen, Christof Koch, Pietro Perona[2]

Attention plays a critical role in modulating visual information that eventually reaches our visual awareness. What can we see when we do not pay attention? Change-blindness experiments have demonstrated that one can be "blind" even to changes in major aspects of a natural scene. The only tasks that need minimal or no attention appear to be those carried out in the early stages of the visual system. Contrary to this common belief, we report that subjects can rapidly detect highly variable objects (e.g., animals or vehicles) in briefly presented novel natural scenes while simultaneously performing another attentionally demanding task (a five-letter form discrimination task). By comparison, they are unable to detect large 'T's from 'L's or a bisected two-color disk from its mirror image under the same condition. This ability does not depend on the fact that our subjects are extensively trained on this type of categorization task. Some of the subjects learn to perform a dual-task involving a given target category (e.g., "animal" or "vehicle") and are subsequently tested using another target category. The pattern of results obtained in that case is comparable. We conclude that some visual tasks associated with "high-level" cortical areas may proceed in the near absence of attention.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*
[2]*Division of Engineering and Applied Science, California Institute of Technology*

394. Face-gender discrimination in the near absence of attention

Leila Reddy[1], Patrick Wilken, Christof Koch

Recent work in our laboratory has shown that rapid natural-scene categorization (animal vs. non-animal) can be done with little or no attentional cost (see abstract by Li, *et al.*). For this study, we investigated the attentional cost associated with a task requiring fine discrimination of stimuli sharing similar features: discrimination of face gender. Subjects were required to perform a face gender discrimination task (face database obtained from MPI, Germany) either alone or concurrently with an attentionally demanding task (five-letter T/L discrimination). Both letters and images were masked following presentation (SOA 160 ms for faces, variable for letters, ranging from 173 ms to 200 ms). Overall, performance on face-gender discrimination suffered remarkably little impairment in the dual-task condition compared to the single-task condition. Similar results were obtained using a set of unfamiliar faces and with a group of participants who had received no training on the gender discrimination task. These results challenge the notion that only low-level representations can be accessed outside the focus of attention.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*

395. Pop-out and preattentive processing are not equivalent: Taking apart a common assumption about visual attention

Rufin VanRullen, Lavanya Reddy[1], Christof Koch

Most theories of visual processing assume that a target will pop-out from a field of distractors if targets and distractors can be discriminated preattentively. The distinction between pop-out and serial search is classically inferred from set size effects in visual search tasks, while the attentional requirements of a visual discrimination task are often assessed using dual-task paradigms, by measuring interference from a concurrently performed attentionally-demanding task. In this project we have shown that there is no such equivalence: while simple feature discrimination tasks that can be performed preattentively lead to pop-out in visual search (e.g., color or orientation discrimination), and many tasks that require attention need serial examination in visual search (e.g., rotated L vs. T or red-green vs. green-red patches), other tasks do not trigger pop-out even though they can be performed preattentively (natural scene categorization, color-orientation conjunction discrimination). Furthermore, certain targets that pop-out among distractors need attention to be effectively discriminated when presented in isolation (rotated L vs. +, cubes with different illumination directions). In other words, the distinctions "pop-out vs. serial search" and "pre-attentive vs. attentive processing" can be independent. We suggest that (i) attentional requirements depend on the existence of specific neuronal populations selective to the target and distractor categories, independent of the level of processing involved (from V1 to IT), while (ii) set size effects in visual search are mainly determined by the level of complexity and the locus of the discrimination; pop-out occurs for targets that are discriminated in early areas (V1-V2) and not in higher areas (V4, IT) due to a combination of two related factors: receptive field size (leading to competitive interactions in higher-level visual areas) and non-classical receptive field interactions (e.g., grouping in lower-level areas).

[1]*Undergraduate student, California Institute of Technology*

396. The time course of attentional selection among competing locations

Fred Hamker

It is still a matter of debate whether observers can attend simultaneously to more than one location. Using essentially the same paradigm as was used by Bichot *et al.* (1999) to show that attention can be "split" among two separate locations, we demonstrate that their previous findings only reflect intermediate stages of (incomplete) attentional selection. Our subjects were asked to discriminate the shapes (circle or square) of two oddly colored targets within an array of eight stimuli. After a certain SOA, eight letters were flashed at the previous stimulus locations, followed by a mask. For a given SOA, the performance of subjects at reporting letters in each location was taken to reflect the distribution of spatial

attention. In particular, by considering the proportion of trials in which none or both of the target letters were reported, we were able to infer the respective amount of attention allocated to each target without knowing, on a trial by trial basis, which location (if any) was receiving the most attentional resources. Our results show that for SOAs around 100-150 ms, attention can be equally split between the two targets, a conclusion compatible with previous reports. However, with longer SOAs, attention ultimately settles at the location of one single stimulus. This is a natural prediction of a computational model of attention (Hamker, 2001) in which the planning of saccadic eye movements guides attentional selection. The results can be accounted for by a model of decision making in which the current output of a "refined" saliency map continuously feeds areas in the fronto-parietal network, which select the unique location of an eye movement by a competition over time. Activity from these areas is fed back continuously to extrastriate visual areas. Thus, the SOA determines the state of this competition at the time the letters were flashed and ultimately the distribution of attention at different locations.

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References

Bichot, N.P., Cave, K.R. and Pashler, H. (1999) *Percep. Psychophys*. **61**:403-423.

Hamker, F.H. (2001) *Soc. Neurosci. Abstr*. **27**:348.10.

397. Hierarchical learning and attention in robot arm control

S.P. Mysore[1], C. Koch, D. Psaltis[2]

The goal of this research is to develop a robust scheme using hierarchical learning and a computational model of attention in order to learn to (physically) solve a complex motor task. The physical testbed for this scheme is a 3-degrees of freedom (dof) planar robotic arm. The particular task to be solved is the Towers of Hanoi task that is further complicated by the presence of obstacles in the workspace of the robotic arm. The aim is for the robotic arm to learn to pick and place disks, to move around meaningfully in the workspace while avoiding obstacles, and to ultimately do all this while achieving a (physical) solution of the task, starting from any initial configuration. It is to be noted that the logical solution of the task is assumed to be given. It is the motion control part of the task that is to be successfully learned. The algorithmic scheme needs to be generalizable (transferable from task to task within a set of similar tasks); the goal being to achieve a scheme inspired by the (planar) motor control behavior in humans. The examples used for learning are to be generated by optimal trajectory and torque planning strategies from robotics literature. A 1-dof robotic arm has been built. Work is in progress to add two more dof. The algorithmic scheme is also being designed, based on the work by Billock (2001).

[1]*Control & Dynamical Systems graduate student, California Institute of Technology*

[2]*Division of Engineering and Applied Science, California Institute of Technology*

Reference

Billock, J.G. (2001) Attentional control of complex systems, Ph.D. thesis, Caltech.

398. Adaptation of current signals with floating-gate circuits

Alberto Pesavento[1]

We continue to work on a new, adaptive spatial-derivative circuit for CMOS image sensors. The circuit removes its offset as a natural part of its operation using a combination of electron tunneling and hot-electron injection to add or remove charge on a floating-gate of an auto-zeroing amplifier. We designed, fabricated and successfully tested a chip with the circuit. Test results show that the circuit reduces the offsets by more than an order of magnitude.

[1]*Electrical Engineering graduate student, California Institute of Technology*

399. The vibrating retina

Ania Mitros[1]

Motion can be essential for vision in some biological systems. Jumping spiders (Salticidae) scan a narrow retina perpendicularly to its long axis to gather information about the full visual field. Microsaccades are essential to human vision. Locusts sway their heads side to side to estimate distance to targets to which they will jump. I spent the summer of 2001 investigating the use of a similar idea in an imager. The imager (or lens or another component along the optical pathway) is vibrated to produce periodic scanning. Each pixel gathers information along a periodic trajectory, thus converting a spatial signal into a temporal waveform. Since each pixel has access to more information than that in a traditional pixel array, each pixel can also be used as a local feature detector and identifier. I tested an imager chip designed to implement this idea. Due to lack of resources, further development was terminated.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*

400. Self-motion estimation

Ania Mitros[1]

The ability to estimate self-motion is a useful one for autonomous robots. However, the capacity to do so in real time is limited on platforms (like robots) that may be limited in power and size of carried load. Many algorithms targeted at accomplishing this task begin by applying the same calculation to each patch of pixels to estimate the existence of salient features. The features then can be followed from one frame to the next, and the motion of each feature between frames is related to the robot's self-motion. Alberto Pesavento implemented a real-time, low-power feature detector that would be ideal

for this purpose, if not for transistor mismatch which caused each feature detector circuit to function too differently for practical usage. I have begun work on a project that would incorporate analog floating gate memory in Alberto Pesavento's circuits to program away the mismatch. The resulting imaging and feature-detecting chip will be the central element in a small, low-power self-motion estimation system.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*

401. Selective attentional modulation of trace vs. delay fear conditioning in C57BL/6N mice

Colm O'Tuathaigh, C.J. Han, David Anderson, Christof Koch

Studies in both eyeblink and aversive conditioning in humans have reported that attentionally demanding tasks can selectively disrupt the development of both the conditioned response as well as the explicit awareness of the CS-US contingency in the trace conditioning paradigm. In the trace conditioning procedure, a decrease in the conditioned response evoked by a CS is observed following an increase in the temporal distance between it and the US, relative to delay conditioned controls where the US immediately follows the CS. The aim of the present study was to examine whether a similar disruption of trace but not delay fear conditioning in mice could be demonstrated following attentional manipulations analogous to those employed in human studies. In both the delay and trace conditions, animals received six tone CS-footshock US pairings, except that an 18 sec. trace interval was inserted between the CS and US in the trace condition. The attentionally distracting stimulus was a flashing light presentation, where both the stimulus location and interstimulus interval were varied throughout the conditioning session. We found that the noncontingent presentation of a distracting stimulus during conditioning significantly attenuated learning in the trace but not the delay condition at test, compared to non-distracted controls. Furthermore, it was shown that this disruptive effect could not be attributed to associative interference. The results indicate agreement between animal and human data with respect to attentional modulation of trace conditioning, and suggest that this paradigm may provide a putative animal model of the relationship between attention and awareness.

402. The involvement of the anterior cingulate cortex in trace versus delay fear conditioning demonstrated by lesion and c-fos mRNA *in situ* hybridization

C.J. Han, Laurent van Trigt, David J. Anderson, Christof Koch

Attention to the CS-US contingency was previously reported to be necessary for successful trace conditioning, but not for delay conditioning, and the anterior cingulate cortex has been implicated in mediating attention. We examined the involvement of the anterior cingulate cortex in mice using trace versus delay fear conditioning. Trace conditioning is different from delay conditioning in that there is a temporal gap between the end of the conditioned stimulus (CS) and the start of the unconditioned stimulus (US). It has been shown in humans that trace, but not delay, conditioning requires awareness of the CS-US contingency (Clark and Squire, 1998). C57BL/6N male mice were housed in the training chambers overnight and fear conditioned on the second day (6 x 16-sec 2k-Hz sine-wave tone followed by a 2-sec 0.5-mA footshock). The temporal gap between the CS and the US in the trace group was 18 sec, while the CS immediately preceded the US in the delay group. The mice were randomly divided into two groups. The first group (one out of four in each condition) was sacrificed after training for quantification of c-fos mRNA (an indicator of neuronal activity). The second group (the remaining three in each condition) was placed into a different context the following day to test the efficacy of the conditioning. The conditioning was found effective in both the delay and trace groups, compared with the US-only group ($p<0.05$). *In situ* hybridization was carried out using a cRNA digoxygenin-labeled probe and design-based stereology was used to quantify the number of c-fos positive cells. Neuronal activity in the brain during delay and trace fear conditioning were compared. The results showed that mice trained in the trace paradigm had significantly more c-fos positive cells in the anterior cingulate cortex than those trained in the delay paradigm. Furthermore, excitotoxic lesion of the anterior cingulate cortex impaired conditioned tone freezing behavior in mice that received trace conditioning, but not those that received delay conditioning.

Reference

Clark, R.E. and Squire, L.R. (1998) *Science* **280**:77-81.

403. Does a concurrent task that activates anterior cingulate cortex (ACC) interfere with aversive trace, but not delay, conditioning in humans?

Naotsugu Tsuchiya[1], Ronald M. Carter, Christof Koch

Previous studies of human conditioning implicated awareness of CS/US relationship as a prerequisite for conditioning. It has been shown that awareness of the CS/US relationship was necessary for eye-blink conditioning in a trace, but not a delay, paradigm. We used a differential aversive conditioning protocol (auditory CSs; 1s long, shock USs; 250 ms, trace period; 3 s, 76 conditioning trials; 4 habituation, 48 acquisition [16 CS+ with US, 8 CS+ only, 24 CS-] and 24 extinction), assessing subjects' degree of awareness of CS-US contingency by postconditioning questionnaires. We evaluated subjects' abilities to be delay or trace conditioned by distracting them via a high/low-load visual attention task. Subjects (Ss) performed a modified Eriksen flanker task at random interval (mean intertrial interval), where they indicated the direction of the target arrow (either up, down, left or right) by a key press as fast and correctly as possible. We manipulated the attentional load

by varying congruency of flankers. Ss in a high attentional load group indicated the direction of the target arrow flanked by either congruent (50% of trials) or incongruent (50%) arrows, while Ss in a low attentional load indicated the target always flanked by neutral symbols. Behaviorally, Ss in the high attentional load group showed slower RT and higher error rate in incongruent trials compared to those in congruent trials (interference around 150 ms) throughout the conditioning session. We expect this interference to be correlated with activity in ACC, as shown by fMRI. Based on mice ACC lesion study, we expected the activation in ACC by the concurrent task would result in less conditioning in trace, but not delay, conditioning. Preliminary data (n=6 for each group) suggests that Ss successfully conditioned in delay paradigm ($p<0.01$), however, Ss didn't conditioned in trace paradigm ($p>0.2$) for both high/low attentional load groups. In delay conditioning, the degree of conditioning was slightly higher in low load group compared to that in high load group.

[1]*Computation and Neural Systems graduate student, California Institute of Technology*

404. Working memory tasks interfere with more complex conditioning protocols

Ronald M. Carter, Constanze Hofstoetter[1,2], Christof Koch

Previous studies of human conditioning implicated awareness of CS/US contingency as a prerequisite for conditioning. Further defining the role of awareness in conditioning, Clark and Squire (1998) showed for differential eye-blink conditioning that awareness of the CS/US relationship was necessary only in a trace but not in a delay paradigm. We present studies on the role of awareness in single-cue fear conditioning (auditory CS and a shock US) using either a delay or trace (3 s trace period) protocol. We evaluated subjects' abilities to condition while distracting them with a working memory task. Some subjects were asked to perform a visually presented numeric 2-back task (press a button whenever the current number matches the one before the previous) during the experiment. While we observed no conditioning in those subjects, performing the 2-back task during the trace conditioning protocol, subjects performing the task during a delay protocol still showed significant conditioning. Subjects not distracted by a task showed significant conditioning in both trace and delay paradigms.

In further control experiments, subjects had to press a button immediately when a predefined number occurred in the observed stream. This 0-back task involves similar visual in- and motor outputs as the 2-back task, but should not require working memory. Preliminary results suggest that this task may interfere less with conditioning. When verbal information on the CS/US relationship is given prior to single cue trace conditioning, significant conditioning was observed for subjects performing either no task or the 0-back task, but not the 2-back task.

[1]*Computation and Neural Systems special graduate student, California Institute of Technology*
[2]*Institute of Neuroinformatics, ETHZ University, Zurich, Switzerland*

Reference

Clark, R.E. and Squire, L.R. (1998) *Science* **280**:77-81.

405. Complexity in neural systems

Whee Ky Ma

An evolutionary trend towards greater brain complexity can only be studied using new quantitative tools. We developed a first-principles information-theoretical measure for process complexity in networks. It is given by the sum of the spatial diversities of ensemble integration strengths. It rewards both collaboration and specialization, the qualitatively desired aspects, while penalizing homogeneities, including randomness; it does not suffer from the drawbacks of earlier measures. We claim that for network processes, only measures based on correlations rather than on causal perturbations make sense. Our hypothesis is that the functional role of complexity, which is not recognized in activation or connectivity patterns alone, is to provide high computational power (reflected in the differentiation) alongside with robust performance. We are testing this hypothesis in neural network simulations and in data from multi-unit recordings. Another possible application is in the diversity-stability debate in ecology, complexity rather than entropy or connectivity may be the determining factor in food-web stability.

406. A framework for consciousness

Francis Crick[1], Christof Koch

We have summarized our work on the neuronal correlates of consciousness in the mammalian forebrain within a common framework expressed in ten succinct working assumptions. These are: 1) the (unconscious) homunculus and the frontal lobes; 2) unconscious, sensory-motor zombie behaviors and consciousness; 3) coalitions of neurons; 4) explicit representations and essential nodes; 5) higher level first; 6) driving and modulating connections; 7) snapshots; 8) attention and binding; 9) styles of firing; 10) the penumbra and meaning. Most of these ideas have been suggested before, but we believe the combination is original. In various publications and in an upcoming book we have summarized the large body of experimental evidence that is compatible with these ideas.

[1]*Salk Institute, La Jolla, CA*

Publications

Billock, J.G., Psaltis, D. and Koch, C. (2001) The match fit algorithm: A testbed for the computational motivation of attention. In: "Lecture Notes in Computer Science," 2074 II, V. Alexandrov, J. Dongarra, B. Juliano, R. Renner, and C.J. Tan, eds., pp. 208-216, Springer, New York, NY.

Billock, J.G., Psaltis, D., Gray, N. and Koch, C. (2001) Learning with an attention-based supervisor. *Fifth Intl. Conf. on Cognitive and Neural Systems,* Boston, MA.

Chang, L., Speck, O., Miller, E.N., Braun, A., Jovicich, J., Koch, C., Itti, L. and Ernst, T. (2001) Neural correlates of attention and working memory deficits in HIV patients. *Neurology* **57**:1001-1007.

Crick, F. and Koch, C. (2002) What are the neuronal correlates of consciousness. In: "Problems in Systems Neuroscience." L . van Hemmen and T.J. Sejnowski, eds., Oxford University Press, New York, NY. In press.

Crick, F.C. and Koch, C. (2002) Consciousness, the neural correlates of (index entry). In: "The Oxford Companion to the Mind," 2nd edition, R.L. Gregory and O.L. Zangwill, eds. In press.

Herzog, M.H., Fahle, M. and Koch, C. (2001) Spatial aspects of object formation revealed by a new illusion, shine-through. *Vis. Res.* **41**:2325-2335.

Herzog, M.H., Koch, C. and Fahle, M. (2001) Shine-through: Temporal aspects. *Vis. Res.* **41**:2337-2346.

Herzog, M.H., Koch, C. and Fahle, M. (2001) Switching binding states. *Vis. Cogn.* **8**:623-636.

Itti, L., Gold, C. and Koch, C. (2001) Visual attention and target detection in cluttered, natural scenes. *Opt. Eng.* **40**:1784-1793.

Jovicich, J., Peters, R.J., Koch, C., Braun, J., Chang, L., and Ernst, T. (2001) Brain areas specific for attentional load in a motion tracking task. *J. Cogn. Neurosci.* **13**:1048-1058.

Koch, C. and Crick, F. (2001) On the zombie within. *Nature* **411**:893.

Koch, C. and Crick, F.C. (2001) The neural basis of consciousness. In: "Intl. Encyclopedia of the Social & Behavioral Sciences," Vol. 4, N. Smelser and P. Baltes, eds., pp. 2600-2604, Elsevier, Oxford, UK.

Koch, C. (2002) A lighthouse for neural modeling. A review of Theoretical Neuroscience by P. Dayan and L. Abbott. *Nature Neurosci.* **5**:195.

Koch, C. and Crick, F.C. (2002) The neuronal basis of visual consciousness. In: "The Visual Neurosciences," L. Chalupa and J.S. Werner, eds., MIT Press, Cambridge, MA. In press.

Krahe, R., Kreiman, G., Gabbiani, F., Koch, C. and Metzner, W. (2002) Stimulus encoding and feature extraction by multiple sensory neurons. *J. Neurosci.* **22**:2374-2382.

Kreiman, G., Fried, I. and Koch, C. (2002) Single-neuron correlates of subjective vision in the human medial temporal lobe. *Proc. Natl. Acad. Sci. USA* **99**:8378-8383.

Li, F.F., VanRullen, R., Koch, C. and Perona, P. (2002) Rapid natural scene categorization in the near absence of attention. *Proc. Natl. Acad. Sci. USA.* **99**:1073.

Niebur, E., Itti, L. and Koch, C. (2002) Modeling visual selective attention. The "Where" Pathway. In: "Models of Neural Networks IV," Van Hemmen, Cowan and Domany, eds., pp. 247-276, Springer Verlag, New York, NY.

Pesavento, A., Horiuchi, T., Diorio, C. and Koch, C. (2001) Adaptation of current signals with floating-gate circuits. *Analog Integr. Circ. Sign. Proc.* **30**:137-147.

Pesavento, A. and Koch, C. (2001) Methods and circuits for focal-plane computation of feature in CMOS visual sensors. In: "Advanced Research in VLSI: Proc. of the 2001 Conference on Adv. Res. in VLSI," E. Brunvand and C. Myers, eds., pp. 238-248, IEEE Computer Society Press.

Pesavento, A. and Koch, C. (2001) A CMOS imager with focal-plane computation for feature detection. In: "Proc. of the 2001 IEEE Internatl. Symp. on Circuits and System" (ISCAS '01), Sydney, Australia.

Rasche, C. and Koch, C. (2002) Recognizing the gist of a visual scene: Possible perceptual and neural mechanisms. *Neurocomputing*. In press.

Rasche, C. (2002) Structural description with classified polygon and surface features and their groupings. *Int. J. Comp. Vis.* Submitted.

Rees, G., Kreiman, G. and Koch, C. (2002) Neural correlates of consciousness in humans. *Nature Rev. Neurosci*. **3**:261-270.

Steinmetz, P.N., Manwani, A. and Koch, C. (2001) Variability and coding efficiency of noisy neural spike encoders. *BioSystems* **62**:87-97.

VanRullen, R. (2002) Visual saliency and spike timing in the ventral visual pathway. *J. Physiol. Paris*. In press.

VanRullen, R. and Koch, C. (2002) Visual selective behavior can be triggered by a feed-forward process. *J. Cogn. Neurosci*. In press.

Zenger, B. and Koch, C. (2001) Flanker effects in peripheral contrast discrimination: psychophysics and modeling. *Vis. Res.* **41**:3663-3675.

Zirlinger, M., Kreiman, G. and Anderson, D. (2001) Amygdala-enriched genes identified by microarray technology are restricted to specific amygdaloid sub-nuclei. *Proc. Nat. Acad. Sci. USA* **98**:5270-5275.

Bing Professor of Behavioral Biology: Masakazu Konishi
Member of the Professional Staff: Eugene Akutagawa
Visiting Associates: Kazuo Funabiki, Lee Moore
Senior Research Fellow: Jose Luis Peña
Research Fellow: Theresa Nick
Postdoctoral Scholars: Yuichiro Hayashi, Sharad Shanbhag
Volunteer: Yasuko Funabiki

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Summary: We continued to study the neural mechanisms of song learning in zebra finches and sound localization in barn owls. Each scientific field has Holly Grails that everyone wants to solve. Dynamic control of auditory feedback during singing was one of such goals in birdsong research. In 1999, Anthony Leonardo developed a clever method to show for the first time that perturbation of auditory feedback affects the maintenance of adult song. A certain pathway within the song control system is thought to mediate the processing of feedback information. The test of this idea was another Holly Grail. Michale Fee of the Bell Telephone Lab and Anthony Leonardo developed a miniature microdrive that could control the movement of multiple electrodes into the brain of zebra finches. Using this method, Anthony showed that the firing pattern of neurons in one of the nuclei in the pathway did not change in response to perturbation of auditory feedback during singing. This finding calls for a new way of thinking about the role of the pathway. Anthony also showed that changing ensembles of neurons control the acoustic structure of song. Other members of birdsong research addressed different questions such as the duration of memory storage in song learning by Yasuko Funabiki and the relationships between song learning and brain auditory responses during sleep by Teresa Nick.

Introduction of new methods has also enabled us to go after some of the Holly Grails in the field of sound localization. Many animals including humans use the difference in the arrival time of sound between the ears to localize sound in the horizontal plane. The neural circuit that processes the interaural time difference (ITD) is hypertrophied in barn owls. When a sound reaches one ear before the other, primary auditory fibers on the leading side fire earlier than those on the lagging side. The ITD detecting circuit delays the leading neural signals such that the neural signals from the two sides arrive simultaneously at special neurons, which respond maximally in response to this synchrony. How these neurons, called coincidence detectors, detect simultaneous arrival of signals has been the most important issue in the study of sound localization. No one could record intracellularly from these neurons *in vivo*. Kazuo Funabiki developed a method that facilitated intracellular recording in the owl's nucleus laminaris, the site of coincidence detectors. Intracellular recording also revealed unexpected mechanisms by which mid-brain auditory neurons form spatial receptive fields. Jose Luis Peña reports here that the threshold of firing of action potentials is set such that only the peak of a spatial receptive field in terms of postsynaptic potentials is converted to spikes. How the threshold is set so appropriately will have to be answered. Another members of the owl team, Ben Arthur showed that space-specific neurons in the owl's map of auditory space were selective for stimuli in which interaural intensity differences vary with both frequency and source location. Sharad Shanbhag introduced a new twist in our approach to sound localization by analyzing how the owl turns its head towards stimuli in space.

407. An efference copy may be used to maintain the stability of adult birdsong

Anthony Leonardo

Zebra finches use auditory feedback to both learn and maintain their songs. Nucleus LMAN of the anterior neostriatum is believed to be crucial for these processes, and is thought to convey an error-correction signal to the motor control system based on the degree of match between the bird's vocalizations and a memorized song template. We measured the activity of individual LMAN neurons while simultaneously manipulating the auditory feedback that birds heard during singing, thus controlling the level of error they could detect in their songs. LMAN neurons were found to produce spikes locked with millisecond precision to specific acoustic features in individual song syllables. This timing precision is comparable to that seen in the motor control neurons that generate the song itself. Furthermore, perturbation of the auditory feedback heard by singing birds had no effect on LMAN spike patterns, suggesting that rather than auditory feedback, nucleus LMAN processes an efference copy of the bird's motor commands. These findings cast a new light on the role LMAN plays in the learning and maintenance of the bird's song.

408. Neuronal ensemble coding of birdsong

Anthony Leonardo

In many models of neural population coding, similar sensory or motor states are represented in the brain by similar neural ensembles. To explore this issue, we measured the activity of large numbers of single neurons in the pre-motor nucleus RA of the singing zebra finch. During singing, individual RA neurons generate precise bursts of action potential spikes. We found that highly similar song elements were typically produced by stereotyped, but uncorrelated ensembles of RA neurons. Small changes in acoustic structure between two syllables were frequently subserved by entirely different ensembles of RA neurons. Furthermore, the neural activity in RA often changed on a time scale an order of magnitude faster than the acoustic structure in the song, causing constant acoustic outputs to be produced by sequences of rapidly changing neural ensembles. Thus there are dynamics

internal to the song control system that occur reliably and precisely yet are not directly correlated with structure in the song itself. These data represent a new type of neural code underlying complex learned behaviors.

409. Modulation of activity states in the zebra finch song system

Teresa Nick

Although humans spend one-third of their lives asleep, we know very little about the state of the brain during this peculiar behavioral state. Using multi-unit recordings in the nucleus HVC (High Vocal Center), we found that the activity of the zebra finch song system increases during sleep as compared to waking. This finding of auditory gating is the converse of what most studies report during sleep: usually sensory activity decreases during sleep. Our data suggest that the observed activity is not purely sensory, but instead results from the sculpting of pre-existing activity by incoming stimuli. Thus, auditory stimulation appears to alter ongoing brain activity in the sleeping songbird. To better understand the mechanism and function of this phenomenon, we are currently working to assess the activity of individual neurons through tetrode recordings in the HVC. With our technique, we can record from individual HVC neurons for at least one week. There are three major subtypes of neurons in HVC: those that project to Area X and appear to be involved in plasticity, those that project to the nucleus Robustus Archistriatalis and command singing, and GABAergic interneurons which are confined within the borders of HVC. We can identify projection neurons and interneurons by their previously described patterns of activity.

We are specifically attacking the question of function by attempting to alter singing behavior through auditory stimulation during sleep. To address mechanism, we have begun lesioning the noradrenergic system, previously suggested to play a role in auditory gating with the neurotoxin DSP-4 while maintaining stable tetrode recordings.

410. *In vivo* intracellular recording from the owl's nucleus laminaris neurons using coaxial glass electrodes

Kazuo Funabiki

The owl's nucleus laminaris (NL) neurons detects interaural time differences (ITDs) with an accuracy of few tens of microseconds by acting as coincidence detectors of binaural inputs. However, the biophysical mechanisms involved in this process still remain to be elucidated. *In vivo* intracellular recording in this nucleus is notoriously difficult. To overcome some of the difficulties, we developed a coaxial glass electrode that consisted of an inner sharp electrode (resistance of 70-100Mohm) for recording and an outer patch-shaped electrode as a guide. We recorded 28 binaural and ITD sensitive cells. Some of these were outgoing axons of NL neurons, because injection of positive or negative current did not change their excitability. In 15 out of the 28 cells, current injection from the recording electrode changed the cell's excitability. Resting membrane potentials of NL neurons ranged from -65 to -45 mV and spike height ranged from 15mV to 8mV. The width of spikes at half height was about 350-500μs. In all the cells recorded, favorable ITDs and not unfavorable ITDs induced small oscillatory potentials whose frequency was similar to the stimulus tone. This oscillatory component changed its amplitude with ITD, and the change was correlated with the spike number induced by sound.

411. From postsynaptic potentials to spikes in the genesis of auditory spatial receptive fields

José Luis Peña

Space-specific neurons in the owl's inferior colliculus respond only to a sound coming from a particular direction, which is equivalent to a specific combination of interaural time difference (ITD) and interaural level difference (ILD). Comparison of subthreshold psps (postsynaptic potentials) and spike output for the same neurons showed that receptive fields measured in psps were much larger than those measured in spikes in both ITD and ILD dimensions. Space-specific neurons fire more spikes for a particular ITD than for its phase-equivalents (ITD ± 1/F, where F is best frequency). This differential response was much less pronounced in psps. The two sides of pyramid-shaped ILD curves were more symmetrical in spikes than in psps. Furthermore, monaural stimuli that were ineffective in eliciting spikes induced subthreshold psps. The main cause of these changes between psps and spikes is thresholding. The spiking threshold did not vary with the kind of acoustic stimuli presented. However, the thresholds of sound-induced first spikes were lower than those of later sound-induced and spontaneous spikes. This change in threshold may account for the sharpening of ITD selectivity during the stimulus. Large changes in receptive fields between psps and spikes also occur in other animals, sensory systems, and brain areas, suggesting the existence of general principles of conversion from postsynaptic potentials to spikes.

412. Selectivity of space-specific neurons for spectral IID cues

Ben Arthur

Barn owls use interaural intensity differences (IID) to localize sounds in the vertical plane. At a given elevation the magnitude of the IID cue varies with frequency, creating an IID spectrum of cues that are characteristic of that direction. To test whether space-specific cells are sensitive to spectral IID cues, pure-tone IID tuning curves were taken at multiple different frequencies for single neurons in the external nucleus of the inferior colliculus (ICx). For a given neuron, the IIDs eliciting the maximum response (the best IIDs) changed with the frequency of the stimulus by a maximum of 9.4 +/- 6.2 dB. Though such shifts were expected, they are small compared to the 19 +/- 8.8 dB average widths of pure-tone IID tuning curves. Nevertheless, when an

"adaptive" two-tone stimulus was synthesized, in which the IID of each frequency was chosen to match the best IIDs from the pure-tone data, the spike rate significantly increased compared to the "flat" (i.e., non-adaptive) stimulus by 17% for a 10 dB difference in best IIDs. Furthermore, when a two-tone stimulus was synthesized in which one frequency was fixed at its best IID and the second swept in IID, there was a central sharply peaked region of IIDs in which the interaction was facilitory, surrounded by regions of suppression. Collectively, these data show that space-specific neurons are sensitive to spectral IID cues and, hence, suggest that behaving barn owls might use such cues to precisely localize sounds.

413. Three-dimensional kinematics of orienting head responses of the barn owl (*Tyto alba*): Does the owl's head act like the primate eye?

Sharad J. Shanbhag

Donders's law of eye movements states that there exists a unique orientation of the eyes in the head for every gaze direction. Listing's law places a further constraint on Donders's law by specifying that any deflection of the eye from reference position may be described by a single rotation, and that the axes of these rotations lie in a plane. Studies of saccadic eye movements in primates have found that eye positions, measured with respect to the head, generally follow Listing's law. The barn owl (*Tyto alba*) has an extremely limited oculomotor range of 3° and therefore relies on head movements to orient its gaze. The owl's orienting head movements in response to auditory stimuli have speed and trajectory characteristics that resemble closely those of saccadic eye movements. Furthermore, the brainstem and midbrain structures that mediate the control of the owl's head have strong homologs in the mammalian brain, both in connectivity and in functional characteristics. Thus, I have examined the kinematics of orienting head movements of the owl to determine if the laws of Donders, Listing, or both are followed. Head orientation data (azimuth, elevation, and roll angles) were recorded from owls trained to orient to sounds presented in the dark. Expressed as quaternions, the head orientation data could not be described by a planar surface, indicating that Listing's law does not apply to orienting gaze shifts of the barn owl. However, since the head orientations were well fit by a curved surface, the owls' head movements did obey Donders's law. The twist in this surface resembles that described for the primate head during eye-head gaze shifts. This suggests a common underlying neural mechanism of gaze control in the owl and in primates.

Publications

Egnor, S.E.R. (2001) Effects of binaural decorrelation on neural and behavioral processing of interaural level differences in the barn owl (*Tyto alba*). *J. Comp. Physiol*. [A] **187**:589-595.

Nick, T.A. and Konishi, M. (2001) Dynamic control of auditory activity during sleep: Correlation between song response and EEG. *Proc. Natl. Acad. Sci. USA* **98**:14012-14016.

Peña, J.L. and Konishi, M. (2002) From postsynaptic potentials to spikes in the genesis of auditory spatial receptive fields. *J. Neurosci.* **22**:5652-5658.

Peña, J.L., Viete, S., Funabiki, K., Saberi, K. and Konishi, M. (2001) Cochlear and neural delays for coincidence detection in owls. *J. Neurosci*. **21**:9455-9459.

Saberi, K., Takahashi, Y., Egnor, R., Farahbod, H., Mazer, J. and Konishi, M. (2002) Detection of large interaural delays and its implication for models of binaural interaction. *J. Assoc. Res. Otolaryngol.* **3**:80-88.

Takahashi, Y. and Konishi, M. (2002) Manipulation of inhibition in the owl's nucleus laminaris and its effects on optic tectum neurons. *Neuroscience* **111**:373-278.

Lawrence A. Hanson Jr. Professor of Biology and Computation and Neural Systems: Gilles Laurent
Visiting Associate: Chun-Fang Wu[1]
Senior Research Fellow: Mark Stopfer
Postdoctoral Scholars: Glenn Turner, Rachel I. Wilson
Graduate Students: Alejandro Bäcker, Bede M. Broome, Stijn Cassenaer, Shabnam Sarah Farivar, Ron Jortner, Ofer Mazor, Javier Perez-Orive, Valentin Zhigulin
Research and Laboratory Staff: Karen Heyman, Sue Z. Smith
[1]*Professor, Department of Biological Sciences, University of Iowa*

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Summary: We are interested in information coding in the brain and in the design principles of circuits involved in processing sensory information. We are particularly interested in understanding the role of time, synchronization and oscillations in information coding and in relating the biophysical properties of neurons and synapses to the function of the networks in which they are embedded. We therefore study the cellular, synaptic and network aspects of neural processing, and focused our research this year on four different model systems: the olfactory system of insects (antennal lobes and mushroom bodies, circuits analogous to the vertebrate olfactory bulbs and anterior/posterior piriform cortices); the olfactory bulb of rats; the olfactory bulb of zebrafish; and the motion sensitive part of the visual system of insects. Our work combines experimental (behavioral and electro-physiological) and modeling techniques and aims at understanding functional aspects of brain circuits design and the rules of information coding used by the nervous system.

414. Encoding complex odor plume features across ensembles of locust antennal lobe neurons

Mark Stopfer, Gilles Laurent

In previous work we found that odors evoke complex sequences of activity and oscillatory synchronization in ensembles of antennal lobe neurons, encoding information about odor identity and concentration. This encoding mechanism can be evoked either by controlled "square-pulses" of odorant or by turbulent odor plumes in a wind tunnel. In recent work, to explore the principles underlying the encoding of odor plume information, we delivered to each preparation: (1) odors as chaotic, turbulent plumes; and (2) the same odors as pulses of controlled concentration, duration, and interval. During both types of odor presentation we made simultaneous extracellular recordings from ensembles of up to 25 projection neurons (PNs), and from mushroom body Kenyon cells. Additionally, to monitor windborne odor filaments near the locust's intact antenna, we recorded an electroantennogram (EAG) from a second, isolated antenna wired to a DC amplifier. Preliminary results indicate: brief (100 m sec), controlled odor pulses evoked, in the EAG, deflections of amplitude proportional to concentration, and in PNs, complex, consistent firing patterns (enduring about 2 sec), as observed for longer-duration odor pulses. These response patterns varied substantially with both odor identity and concentration. Ensemble responses to brief odor pulses at different concentrations were used to construct response templates for each odorant. By cross-correlating these templates with the PN ensemble response evoked by an odor plume, we could reconstruct the prominent odor plume features evident in the EAG. The identity, concentration, and temporal features of the odor plume thus appear to be encoded as complex, time-varying firing patterns across ensembles of PNs.

415. Odor identity and concentration are encoded by temporal activity patterns across ensembles of neurons in the locust antennal lobe

Mark Stopfer, Vivek Jayaraman, Gilles Laurent

Odors evoke complex sequences of activity in antennal lobe projection neurons (PNs). These slow temporal patterns, evolving over hundreds of msec and consistent from trial to trial, have been shown to contain information about odor identity. To examine how odor concentration is encoded, we delivered five order-of-magnitude concentrations of three odors while making extracellular recordings from ensembles of up to 15 PNs. Most PN response patterns changed substantially with concentration, often in seemingly unpredictable ways. Hypothesizing that information about both odor identity and concentration was encoded at the level of the neuronal ensemble, we analyzed ensemble responses over time using a nonlinear dimensionality reduction techniques, Principle Component Analysis (PCA) and Locally Linear Embedding (LLE, Roweis and Saul, 2000). LLE recovers the global structure of high-dimensional input (here, time-binned response patterns in multiple PNs) by computing low-dimensional, neighborhood-preserving embeddings of the input. In five of five experiments we could reliably separate three-dimensional embeddings into odor clusters and these into concentration sub-clusters. This clustering was robust to changes in both PCA and LLE parameters. The LLE technique proved more useful for separating odor identity and concentration clusters, suggesting that this information lies along complex, non-linear manifolds. We tried separating the clusters with spatial and firing rate

information alone by using ensembles of averaged firing rates. Such clustering proved impossible for three-dimensional embeddings, suggesting that information about odor concentration, as well as identity, is encoded within the temporal firing patterns of ensembles of PNs.

Reference

Roweis, S.T. and Saul, L.K. (2000) *Science* **290**:2323.

416. Inhibitory interneurons and olfactory representations in the mushroom body

Glenn C. Turner, Gilles Laurent

We are investigating the role of inhibitory interneurons in shaping olfactory representations in the mushroom body (MB), the memory center of the locust brain. We have identified a single giant GABAergic neuron (GGN) that is well suited to implement feedback inhibition in the MB, as it extends neuritis throughout the input (calyx) and output (lobes) regions of this neuropil. We are testing the hypothesis that the GGN serves as a gain-control element for activity in the MB, reading out the total/summed Kenyon cell (KCs, the intrinsic MB neurons) excitation in the lobes and sending a proportional amount of inhibition to the KC dendrites in the calyx. Such an adaptive gain-control mechanism would be useful, for example, to ensure that changes in the degree of excitation in the antennal lobe brought about by different concentrations of odorant would not overly distort the mnemonic representation of the odor. The GGN does not fire action potentials; rather it responds to odors with sharp oscillations, possibly calcium spikelets or periodic compound EPSPs, which are superimposed on a prolonged depolarization. The cell's oscillatory activity is phase locked to the oscillatory activity of the PN population, and is roughly 180 out of phase with the preferred phase at which KCs spike. This suggests that the strength of the GGN response on one cycle will modulate the probability of KC firing on the next cycle. Moreover, the GGN's response correlates with the strength of the olfactory stimulus: the higher the odorant concentration, the greater the response amplitude and duration. These observations are consistent with the hypothesis that the GGN helps to maintain odor representations in the MB in a format that is independent of odorant concentration. To directly test this proposal, we plan to examine MB odor representations using multiunit Kenyon-cell recordings before and after inactivating the GGN.

417. Pharmacology of antennal lobe synapses

Rachel I. Wilson, Gilles Laurent

Projection neurons (PNs) of the locust antennal lobe (AL) respond to odors with complex patterns of activity. These patterns are generally characterized by bursts of action potentials separated by periods of deep hyperpolarization lasting hundreds of milliseconds (Laurent and Davidowitz, 1994). These hyperpolarizing epochs are likely to reflect the complex dynamics of synaptic inhibition arising from the antennal lobe network (Laurent, *et al.,* 1996; Bazhenov, *et al*., 2001). However, these patterns are not affected by the $GABA_A$ antagonist picrotoxin, suggesting that a non-$GABA_A$-type inhibitory receptor may be involved (MacLeod and Laurent, 1996). Different odors elicit different slow patterns in a given PN, and different PNs respond to the same odor with different patterns (Laurent and Davidowitz, 1994). Therefore, slow patterns contain information about odor identity that may be used by downstream neurons to discriminate between similar odors. We would like to test this hypothesis by pharmacologically blocking slow IPSPs in antennal lobe PNs, and then examining the effect of this manipulation on: (1) an ideal observer's ability to classify PN responses based on odor stimulus, using a clustering algorithm (MacLeod and Laurent, 1996); and (2) an insect's ability to perform a behavioral odor-discrimination task (Stopfer *et al*., 1997).

These experiments require pharmacological characterization of AL synapses. We have begun by developing a semi-reduced preparation that permits whole-cell patch clamp recording from AL neurons and efficient exchange of drugs in superfused saline. A single shock to a stimulating electrode placed in the AL neuropil typically elicits four types of postsynaptic currents in a voltage-clamped PN.

We also plan experiments to probe AL synaptic connectivity using simultaneous intracellular recordings from pairs of AL neurons. Recordings from connected PN/PN pairs, PN/LN pairs, and LN/LN pairs will be used to confirm which population(s) of afferents give rise to each of the four synaptic conductances. Minimal stimulation of the antennal nerve using a suction electrode can also be used to activate ORN-PN and ORN-LN synapses. These experiments will also reveal the probability of synaptic connectivity between each cell type, which should facilitate realistic computational modeling of the AL (Bazhenov *et al*., 2001).

References

Bazhenov, M., Stopfer, M., Rabinovich, M., Abarbanel, H.D.I., Sejnowski, T.J. and Laurent, G. (2001) *Neuron* **30**:569-81.

Laurent, G. and Davidowitz, H. (1994) *Science* **265**:1872-1875.

Laurent G., Wehr, M. and Davidowitz, H. (1996) *J. Neurosci.* **16**:3837-3847.

Leitch, B. and Laurent, G. (1996) *J. Comp. Neurol.* **372**:487-514.

MacLeod, K. and Laurent, G. (1996) *Science* **274**:976-979.

Stopfer, M., Bhagavan, S., Smith, B.H. and Laurent, G. (1997) *Nature* **390**:70-74.

418. The olfactory system is invariant to odorant volatility

Alex Bäcker

It is sometimes held that volatility is a primary determinant of our ability to smell any given compound. Sometimes, corrections are even made for volatility when comparing olfactory responses to odorants at any given concentration in solution (Brockerhoff and Grant, 1999).

These corrections do not significantly reduce the dynamic range of the amplitude of olfactory responses, though. I showed that if the odorant source is a solution whose composition is similar to that of the mucosa covering olfactory receptors, and in equilibrium, the system is invariant to the volatility of the odorant, i.e., two compounds present in a solution in equal activities will reach the olfactory mucosa in equal activities even if one is significantly more volatile than the other. This is due to the fact that the solubilization process is the inverse of the evaporation process if the activity coefficients of the odorants are similar for the source and the mucosa, and their strong dependencies on volatility therefore cancel each other out. Furthermore, even when the compositions are not similar, odorant activity at the mucosa is not an explicit function of the volatility, and depends only on the activity coefficients of the odorant at the source and the olfactory mucosa-the gas phase acts merely as a carrier. I extended the analysis to solid odor sources, for which the same concept holds. Interestingly, the same holds not only for noses whose receptors are immersed in solution, but also for artificial noses for which odorants are detected by sorption into a polymer phase.

Reference

Brockerhoff, E.G. and Grant, G.G. (1999) *J. Chem. Ecol.* **25**:1353-1367.

419. Gain control in early olfactory circuits

Alex Bäcker

It has been known for some time that an increase in odor concentration causes a monotonic increase in the active area of the vertebrate olfactory bulb and insect antennal lobe as measured with calcium imaging, suggesting that downstream targets may have to deal with a large dynamic range of activity levels. On the other hand, response of mitral cells to increasing odor concentration has traditionally presented a less clear picture, with non-monotonic changes in firing rate. How are these pictures of input and output reconciled? I recorded intracellularly from locust PNs and showed that the mean firing rate across PNs is relatively invariant to odor concentration, due to a dynamic balance of excitation and inhibition acting as a gain control: as odor concentration increases, inhibition is strengthened in parallel to excitation. This effect, which is missed by calcium imaging techniques, may provide a solution to the conundrum of how complex odor blends do not evoke the percepts corresponding to all of their components, but rather a different percept altogether (Laing and Francis, 1989), despite the fact that receptors' responses are monotonic in concentration: the increase of inhibition with increasing concentration may allow detectors to be inhibited with the addition of additional blend components, allowing for cells to be tuned to particular odors. These may constitute dual roles of slow inhibition, whose role is controversial (Laurent, 2000), in the antennal lobe: tuning PN responses to respond specifically to particular odors, exhibiting lower responses to supersets of their preferred stimuli, and gain modulation, to keep PN firing rates within PNs' dynamic range and that of the decoders.

References

Laing, D.G. and Francis, G.W. (1989) *Physiol. Behav.* **46**:809-814.

Laurent, G. (1999) *Science* **286**:723-728.

420. A computerized odor delivery system for arbitrary time-varying concentrations and mixtures

Alex Bäcker

The behavior of a sensory system is only as rich as the set of stimuli it is faced with. Sensory physiologists are thus faced with the challenge of generating a set of stimuli as rich as possible in a controllable manner. As compared to vision and hearing, where computer screens and synthesizers provide great control and flexibility, the study of olfaction has suffered from a relative lack of flexible odor delivery systems. I developed a computerized odor delivery system capable of delivering arbitrary concentrations controlled in real-time, binary mixtures in arbitrary ratios, and the potential to deliver arbitrary discrete or continuous stimulus waveforms.

421. Characteristics of sleep in insects

Bede Broome

The function and mechanisms of sleep remain elusive for neurobiologists despite numerous studies. There is strong evidence that sleep has a restorative function and many suggestive results that sleep has a role in learning and memory. The precise natures of these phenomena are unknown. Mounting evidence suggests that sleep occurs in all animals, including insects, which offer special experimental advantages. Recent studies have documented sleep-like behavior in *Drosophila melanogaster.*

However, without understanding the neurological activity associated with sleep in insects it will be impossible to say if their sleep is truly analogous to ours. The goal of this study is to identify an electrophysiological signature for sleep in locusts, large insects amenable to electrophysiology. Specifically, neuronal activity will be correlated with behavioral assays, and pharmaceuticals affecting sleep will be analyzed. Furthermore, state-dependent differences in sensory processing will be monitored. These studies will employ extracellular and intracellular recording techniques along with several behavioral and physiological monitoring systems. Coordinated neuronal assemblies and their state dependency will be analyzed. The ultimate goal is to establish a strong foundation for an insect sleep model.

422. Associative learning and sensory representations

Stijn Cassenaer

Odor representations have been characterized extensively in the locust antennal lobe (AL), where they are carried by assemblies of projection neurons (PNs) that

evolve reliably over time, both in terms of firing rate and extent of phase-locking to the local field potential (Laurent *et al*., 1996). Their decoding by Kenyon cells in the mushroom body (MB) has been studied recently as well (Perez-Orive *et al.,* 2002). In sharp contrast to the dense, redundant representations of the AL, odor representations in the MB were found to be very sparse and carried by very selective neurons. There is evidence to suggest that both of these brain regions are involved in associative learning (Hammer and Menzel 1998; Faber *et al*., 1999; McGuire *et al*., 2001). We want to determine how odor representations are affected by the formation of new associative memories, that is, investigate how associative memories are implemented in these networks.

An identified honeybee neuron, VUMmx1, appears to encode the reward stimulus in conditioning experiments (Hammer, 1993). Briefly, the evidence for this claim consists of four main findings: the cell responds to the unconditioned stimulus (US, typically sucrose) by increasing its firing rate. Activation of the cell by depolarization can replace the US when paired with odors in conditioning experiments. Injection of its neurotransmitter, octopamine (OA), in the target structures (MB or AL) can also replace the US. Lastly, the temporal requirements for activation of the cell (or OA injection) and delivery of the US for conditioning appear to be similar.

In order to examine the interaction between associative learning and odor representations, we employ the locust equivalents of this neuron (DUM SA 1 and 5; Braunig, 1991). By pairing odors with the putative neural representation of reward, we are attempting to induce the formation of associative memories. Using tetrode recordings, we found that pairing of odors with the injection of OA alters the population response in the antennal lobe, as compared to saline-injection controls. To further examine how odor representations in the AL and MB networks are changed, we want to pair odors with depolarizing current injection of the DUM neurons, while monitoring the effect with tetrodes in the AL and MB. To determine the intracellular and synaptic changes underlying the network effects in the AL we will carry out simultaneous intracellular PN or LN and DUM recordings.

References

Braunig, P. (1991) Phil. Trans. Royal Soc. London Series B-Biol. Sci. **332**:221-240.

Faber, T. *et al.* (1999) *Nature Neurosci.* **2**:74-78.

Hammer, M. (1993) *Nature* **366**:59-63.

Hammer, M. and Menzel, R. (1998) *Learn. Memory* **5**:146-156.

Laurent, G. *et al*. (1996) *J. Neurosci.* **16**:3837-3847.

McGuire, S.E. *et al.* (2001) *Science* **293**:1330-1333.

Perez-Orive, J. *et al.* (2002) *Science* **297**:359-365.

423. Probing the role of β-lobe neurons in olfaction

Sarah Farivar, Gilles Laurent

The insect mushroom body (MB) receives and processes olfactory information. The MB is a highly conserved structure, found in all but a few insect species– and has been shown to be a relevant area in learning and memory of olfactory information. The morphology of the intrinsic cells of the MB, the Kenyon cells (KCs) has been extensively studied, and their integrative properties are starting to be understood. To help decipher their role in odor processing, we are studying their output onto a specific set of target neurons called the β-lobe neurons (βLNs). Using electrophysiology and intracellular dye fills of βLNs, we have begun to characterize the morphology and odor responses of this class of cells and to correlate those properties with those of their KC inputs. Preliminary data suggests that β-lobe neurons seem to fall into several morphological and functional classes, though these classes may not necessarily overlap.

424. Firing statistics and network dynamics of simultaneously-recorded neurons in the locust antennal lobe

Ofer Mazor, Roni Jortner, Christophe Pouzat[1], Gilles Laurent

The antennal lobe (AL) is the first relay in the insect olfactory pathway. The lobe contains two main neuron types: ca. 830 projection neurons (PNs) and ca. 300 inhibitory local neurons (LNs). The response of the AL to an olfactory stimulus is an odor-specific spatio-temporal pattern of PN activity, involving over half of the PN population. In this study, we examine the spontaneous activity of this system to better understand the internal dynamics that underlie the odor-evoked behavior.

We can routinely record 5-25 PNs simultaneously for several hours using silicon multi-electrode probes. We begin by showing that, at rest, the continuum of firing properties (from apparent burstiness to tonic firing) observed across PNs can be modeled as a renewal process, using only five parameters. Given this description of single PN behavior, we can detect and test the significance of interactions among simultaneously recorded PNs. We find that even at rest, there are significant positive and negative correlations between most pairs of PNs and that these correlations are present over a wide range of timescales (1 ms – 30 s). Furthermore, we typically find clusters of positively-correlated PNs with negative correlations across clusters.

Finally, we perform intracellular recordings of single LNs simultaneously with the PN recordings. These experiments allow us to observe the interactions between these two types of neurons. For example, during periods of spontaneous activity, we find both PN-LN pairs with strongly correlated activity and other pairs with no such correlations. In addition, by driving the LNs with a current injection, we hope to determine the direct extent of influence of an individual LN on the population of PNs.

[1]*Laboratoire de Physiologie Cérébrale, Paris, France*

425. Encoding and decoding with oscillations in the insect olfactory system

Javier Perez-Orive, Ofer Mazor, Glenn C. Turner, Stijn Cassenaer, Rachel I. Wilson, Gilles Laurent

In the insect olfactory system, oscillatory synchronization appears to be functionally relevant (Stopfer *et al*., 1997) and reflects the coherent activation of dynamical neural assemblies (Laurent *et al*., 1996). We examined the role of such oscillatory synchronization in the transfer of information between networks in this system. The source, called the antennal lobe, is the obligatory relay for olfactory afferent signals and generates the oscillatory output. The target, called the mushroom body, is responsible for the formation and retrieval of olfactory memories (Zars *et al.,* 2001). We found that the format of odor representations differs dramatically across these structures. While representations are dense, dynamic and seemingly redundant in the antennal lobe, they are sparse, brief and carried by very specific neurons in the mushroom body. This transformation relies on a combination of oscillatory synchronization, feed-forward inhibition, connectivity patterns and biophysical properties. These mechanisms act together to selectively filter and synthesize the output from the antennal lobe. These results (Perez-Orive *et al*., 2002) provide direct support for the functional relevance of correlation codes and shed some light on the role of oscillatory synchronization in sensory networks.

References

Laurent, G., Wehr, M. and Davidowitz, H. (1996) *J. Neurosci*. **16**:3837-3847.

Perez-Orive, J., Mazor, O., Turner, G., Cassenaer, S., Wilson, R. and Laurent, G. (2002) *Science* **297**:359-365.

Stopfer, M., Bhagavan, S., Smith, B. and Laurent, G. (1997) *Nature* **390**:70-74.

Zars, T., Fischer, M., Schulz, R. and Heisenberg, M. (2000) *Science* **288**:672-675.

426. Electroconvulsive response in the locust

Chun-Fang Wu[1], Bede Broome

In *Drosophila*, it has been shown that high-frequency electroconvulsive stimuli applied across the brain evoke a stereotypic seizure repertoire, consisting of several bouts of spasm interposed with paralysis. Corresponding motor output, i.e., spike bursting activity, can be recorded from different muscle fibers and across different body axes. Interestingly, this fixed-action pattern behavior resembles many aspects of seizure in vertebrate species. A genetic analysis of seizure susceptibility would be facilitated by using this stereotypic electroconvulsive activity, as it could be modified by mutations that affect nerve excitability and synaptic transmission (Lee and Wu, 1998, 2000).

It will be interesting to determine whether electroconvulsive stimuli can induce similar seizure activity in the locust, a preparation well suited for detailed electrophysiological studies of neural circuit properties. We found that the same electroconvulsion protocol used in *Drosophila* (200-Hz high voltage pulses) cause overt behavioral seizure and heightened electromyogram signals in the locust. These activities consist of recurrent spontaneous discharges of nerve and muscle units detectable in muscles of the abdomen, the thorax and the base of antennae. Further experiments will characterize the threshold of seizure induction, muscle-specific seizure patterns, activities of the underlying neural circuits, and their pharmacological sensitivity.

[1]*Professor, Dept. of Biological Sciences, Univ. of Iowa*

References

Lee J. and Wu C.-F. (1998) *Soc. Neurosci*. Abstr **24**:16.

Lee J. and Wu C.-F. (2000) *Soc. Neurosci*. Abstr **26**:221.

Publications

Bazhenov, M., Stopfer, M., Rabinovich, M., Huerta, R., Abarbanel, H.D.I., Sejnowski, T.J. and Laurent, G. (2001) Model of transient oscillatory synchronization in the locust antennal lobe. *Neuron* **30**:553-567.

Bazhenov, M., Stopfer, M., Rabinovich, M., Huerta, R., Abarbanel, H.D.I., Sejnowski, T.J. and Laurent, G. (2001) Model of cellular and network mechanisms for temporal patterning in the locust antennal lobe. *Neuron* **30**:569-581.

Friedrich, R. and Laurent, G. (2001) Dynamical optimization of odor representations by slow temporal patterning of mitral cell activity. *Science* **291**:889-894.

Gabbiani, F., Krapp, H.G., Koch, C. and Laurent, G. (2002) Multiplicative computation by a looming-sensitive neuron. *Nature*. In press.

Gabbiani, F., Mo, C. and Laurent, G. (2001) Invariance of angular threshold computation in a wide-field looming sensitive neuron. *J. Neurosci.* **21**:314-329.

Laurent, G. (2002) Olfactory network dynamics and the coding of multidimensional signals. *Nature Rev. Neurosci.* **3**. In press.

Laurent, G., Stopfer, M., Friedrich, R., Rabinovich, M.I., Volkovskii, A. and Abarbanel, H.D.I. (2001) Odor encoding as an active, dynamical process: Experiments, computation and theory. *Ann. Rev. Neurosci.* **24**:263-297.

Nusser, Z., Kay, L.M., Laurent, G., *et al*. (2001) Disruption of GABA(A) receptors on GABAergic interneurons leads to increased oscillatory power in the olfactory bulb network. *J. Neurophysiol*. **86**:2823-2833.

Perez-Orive, J., Mazor, O., Turner, G., Cassenaer, S., Wilson, R. and Laurent, G. (2002) Oscillations and sparsening of odor representations in the mushroom body. *Science* **297**:359-365.

Pouzat, C., Mazor, O. and Laurent, G. (2002) Model-based spike-sorting: An automated procedure for classification with confidence intervals. *J. Neurosci. Meth.* In press.

Rabinovich, M.I., Volkovski, A., Lacanda, P., Huerta, R., Abarbanel, H.D.I. and Laurent, G. (2001) Dynamical encoding by networks of competing neuron groups: Winnerless competition. *Phys. Rev. Lett.* **87**:68102-1,4.

Professor of Biology: Shinsuke Shimojo
Visiting Associate: Lynne E. Bernstein[1], Romi Nijhawan[2]
Visitor: Fumiko Maeda[3]
Senior Research Fellow: Ladan Shams
Postdoctoral Scholars: Joydeep Bhattacharya, Christine Chee-Ruiter, Ryusuke Hayashi, Bhavin Sheth, Carol Yin
Graduate Students: Ryota Kana[4], Patricia Neal, Dylan Nieman, Claudiu Simion, Daw-An Wu
Undergraduate Students: Yile Ding, China Hanson, Alice Lin, Richard Spjut, Samuel Thompson, Rui Wang
Research and Laboratory Staff: Karen Heyman, Benjamin Matthews, Rory Sayres, Sue Z. Smith
[1]*House Ear Institute, Los Angeles, CA*
[2]*University of Sussex, Falmer, Brighton, UK*
[3]*UCLA Medical School, Los Angeles, CA*
[4]*University of Utrecht, The Netherlands*

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Summary: We continue to examine the dynamic/adaptive nature of human visual perception—including its cross-modal, representational, sensory-motor, developmental, and neurophysiological aspects. Using methods having a broad scope, we also examine how exactly peripheral sensory stimuli, neural activity in the sensory cortex, and the mental experience of perception are related to each other.

(1) Following our discovery of the "illusory double flash" effect (a briefly presented single flash can be perceived as a double flash), we applied the EP (Evoked Potential) technique and found a physiological counterpart, i.e., non-linear enhancement of visual evoked potentials by accompanying sounds. Further, we applied wavelet analysis to the EP data to find that there is a long-range synchrony in neural activity in the visual cortex corresponding to the illusory flash. In a different situation, repeated sounds when synchronized with repeated visual stimuli turned out to facilitate detection of the visual stimuli ("beating heart" illusion). Likewise, we found that an auditory target (a sound) with motion can make a stationary visual target appear to move. In yet another stimulus condition where the observers should judge whether two moving visual targets are streaming across, or bouncing off each other, somatosensory as well as auditory transient signals turned out to significantly change visual motion perception. We examined how early in human infancy the brain becomes capable of integrating multisensory inputs in an adult-like way. The habituation/dishabituation experiments and eye movement recording results suggest that 5 months on average is the onset age for such a cross-modal ability. These findings altogether strongly indicate that visual information processing is affected by transient signals in other sensory modalities much more vigorously and developmentally earlier than we used to believe, and that the interaction occurs at relatively early cortical levels.

(2) Following our discovery of "cortical afterimage" i.e., afterimage of a cortically filled-in visual surface, we manipulated edge (real/illusory) and color (luminance) information in the stimulus configuration during both adapting and testing periods. The results indicate vigorous interactions among these signals, and the semi-abstract nature of the cortical surface representation.

(3) Microscopic psychophysics of visual perception in a very brief time period (1-200 ms) has revealed how the visual system identifies transient visual input in the context of a sustained, or continuously changing, frame of reference. Based on our earlier work employing this effect (called the "flash lag" effect) as the paradigm, we further studied how a transient visual stimulus is localized with regard to a continuously moving object. Our new findings suggest that grouping of stimuli to construct an object representation takes a significant role in the mechanism underlying the flash lag effect.

(4) Utilizing our technique combining transcranial magnetic stimulation (TMS) and psychophysics, we have further isolated the exact spatio-temporal details of the visual cortical processes that are directly responsible for visibility and its suppression in the domains of both space and time. By applying dual-pulse TMS to the visual cortex, our previous work indicates that the way TMS interacts with visual inputs is different between sustained and transient visual inputs, and that the interactions are surprisingly non-linear. More recently, we found yet another surprising nature of the TMS effect – it can visualize the accurate (i.e., physical location) of a moving target even under conditions in which the target appears elsewhere due to a perceptual illusion. In a variation of the flash lag effect mentioned above, for example, a transient change of a feature (say, the color changing to red momentarily) in the moving object appears ahead in the motion trajectory relative to its physical location. Yet, with the dual-pulse TMS, in addition to this motion-extrapolated red bar, another bar at its physical position becomes visible. While this indicates that the flash lag effect occurs after the initial cortical representation of the space, it also implies dynamic modulation of visibility threshold by TMS.

(5) When an object moves laterally behind an occluder with a vertical slit in it, the entire shape of the object can easily be perceived even when the eyes fixate at the slit so that only a narrow portion of the retina corresponding to the slit is stimulated ("slit view" effect). This provides us with an opportunity to separate retinal stimulus and percept, and to see which of these (i.e., stimulus or percept) neural activity in the visual cortex correlate with. Our fMRI study suggests that activity in

both the human homolog of the area MT (the visual motion area) and the temporal object recognition areas are correlated with rigid shape perception, whereas earlier areas do not show such correlation. The results indicate the presence of dynamic shape re-construction processes in the visual pathway.

(6) We recently developed a new behavioral paradigm to study the intrinsic and dynamic relationship between bodily orienting action and emotional judgments such as "liking." As the baseline finding, we found that the observer's eye gaze is increasingly more towards the stimulus (a face) that the observer would eventually judge as more attractive in about 800-1000ms immediately before the preference response. Thus, it is not only that we see an object more because we like it more, but also that we like it more because we see it more. Our latest study further shows that; (a) there is a component related to selection-memory bias which is common across various tasks including liking, disliking and an emotionally neutral geometric judgment, but it is too weak to explain the overwhelming cascade-like gaze shift in the case of liking, and (b) the cascade effect is not limited to faces only, because an even stronger effect was found with "Fourier descriptors" i.e., mathematically-defined, unfamiliar shapes. The whole conscious events of emotional experience may rely heavily on this type of interaction below the threshold of consciousness. We are now extensively investigating how robust and generic this "cascade" effect is.

(7) Partly motivated by the notorious POKEMON incident that occurred in Japan in December 1997, we compared pupillary responses to color flicker in normal and photo-sensitive subjects. We also measured MEG responses from brains of normal and photosensitive subjects while they were watching such stimuli. The results indicate surprising sensitivity in both types of subjects to chromatic flickers, even when luminance was equated between colors. The MEG results indicate a defensive mechanism located in a parieto-occipital region whose activity is earlier than the later spreading activity from the occipital visual region, not specific to the stimulus frequency, and negatively correlated with the late occipital activity.

(8) Visual coordinate transformations due to eye or visual-frame movements were examined. In particular, we studied gaze-dependent modulation of various sorts of visual aftereffects, and in all that we studied (motion, orientation, size, detection threshold, etc.) we found substantial modulation (up to 20 %). In the most extreme cases, we were successful in producing negative depth (color) aftereffects in the opposite directions simultaneously in a single subject, depending on the gaze direction. These results clearly indicate the presence of gaze modulation in the visual pathways, and its relevance to our visual perception.

We will maintain our efforts, with emphasis on a combination of techniques, a wider variety of subjects, the developmental aspects, and the dynamic/adaptive aspects of processing, to further understand visual perception in its relationship to the motor and other perceptual modality systems.

427. Visual feature binding in early infancy

Gentaro Taga[1], Tomohiro Ikejiri[1], Tatsushi Tachibana[1], Shinsuke Shimojo, Atsuhiro Soeda[2], Keiko Takeuchi[3], Yukuo Konishi[4]

How does the developing brain of the human infant solve the feature-binding problem when visual stimuli consisting of multiple colored objects are presented? A habituation-dishabituation procedure revealed that one-month-old infants have the ability to discriminate changes in the conjunction of a familiar shape and color in two objects. However, this good earlier performance was followed by poorer performance at two months of age. The performance improved again at three months of age. Detailed analysis of the oculomotor behaviors revealed that the age of two months was a period of drastic transition when the tendency to stay with the fixated objects disappeared and repetitive saccades between the two objects emerged. Our findings suggest that the ability to perceive conjunctions of features is available to infants very early, that the perceptual/neural basis at one and at three months of age may be fundamentally different, and that feature integration by vigorous eye movements or selective attention may be the key functional difference between the age groups.

[1]*University of Tokyo, Tokyo, Japan*
[2]*Tokyo Metropolitan Maternity and Child Health Institute, Tokyo, Japan*
[3]*Fukui University, Fukui, Japan*
[4]*Saitama Medical School, Kawagoe, Japan*

Reference

Taga, G., Ikejiri, T., Tachibana, T., Shimojo, S., Soeda, A., Takeuchi, K. and Konishi, Y. (2002) *Perception* **31**:273-286.

428. Perceptual organization of moving stimuli modulates the flash-lag effect

Katz Watanabe[1], Romi Nijhawan[2], Beena Khurana[2], Shinsuke Shimojo

When a visual stimulus is flashed at a given location the moment a second moving stimulus arrives at the same location, observers report the flashed stimulus as spatially lagging behind the moving stimulus (the flash-lag effect). The authors investigated whether the global configuration (perceptual organization) of the moving stimulus influences the magnitude of the flash-lag effect. The results indicate that a flash presented near the leading portion of a moving stimulus lags significantly more than a flash presented near the trailing portion. This result also holds for objects consisting of several elements that group to form a unitary percept of an object in motion. The present study demonstrates a novel interaction between the global configuration of moving objects and the representation of their spatial position and may provide a new and useful tool for the study of perceptual organization.

[1]*Juntendo University School of Medicine, Tokyo, Japan*
[2]*University of Sussex, Falmer, Brighton, UK*

Reference

Watanabe, K., Nijhawan, R., Khurana, B. and Shimojo, S. (2001) *J. Exp. Psychol. Human* **27**:879-894.

429. Afterimage of perceptually filled-in surface

Shinsuke Shimojo, Yukiyasu Kamitani[1], Shin'ya Nishida[2]

An afterimage induced by prior adaptation to a visual stimulus is believed to be due to bleaching of photochemical pigments or neural adaptation in the retina. We report a type of afterimage that appears to require cortical adaptation. Fixating a neon-color spreading configuration led not only to negative afterimages corresponding to the inducers (local afterimages), but also to one corresponding to the perceptually fitted-in surface during adaptation (global afterimage). These afterimages were mutually exclusive, undergoing monocular rivalry. The strength of the global afterimage correlated to a greater extent with perceptual filling-in during adaptation than with the strength of the local afterimages. Thus, global afterimages are not merely by-products of local afterimages, but involve adaptation at a cortical representation of surface.

[1]*Beth Israel Deaconess Medical Center, Harvard Medical School, Cambridge, MA, USA*
[2]*NTT Communication Science Laboratory, Japan*

Reference

Shimojo, S., Kamitani, Y. and Nishida, S. (2001) *Science* **293**:1677-1680.

430. Kanizsa square without pacmen created by selective edge adaptation

Yukiyasu Kamitani[1], Shinsuke Shimojo

A low-contrast disk can be made invisible, and filled in by the surround brightness/color, after adaptation to a high-contrast edge that spatially overlaps with the contour of the disk (Shimojo and Kamitani, VSS 2001). We report here that this edge adaptation technique can be applied to "part of" the edges in the Kanizsa configuration, leading to emergent percept of illusory filled-in surfaces. Low-contrast "packman" stimuli (5% brighter/darker than the gray background) that constituted the conventional Kanizsa square were presented, after adaptation to four high-contrast disks that alternated between white and black at 1 Hz for 15 s. The pacmen and the disks shared their edges except for the wedge portions. Thus the edges corresponding to the corners of the "square" were left unadapted. Subjects fixated to a dot placed in the middle of the four disks/pacmen throughout the adaptation and test periods. All subjects (9) reported that at the beginning of the test period (for 2.7-4.3 s), only a square was perceived on a homogeneous background, and that the square region was filled with a distinct brightness, which was darker (brighter) than the background when the luminance of the pacmen was higher (lower) than the background. A similar illusory square was perceived using different shapes of stimuli that were designed to leave the same edges (the corners of the "square") unadapted, even if the test stimulus (cross- or pie-shaped figures) did not produce a vivid illusory square/surface by itself. The results indicate that the edges (local luminance contrast) at the corners were preserved, and sufficient to create the illusory square, while the other edges were masked by adaptation, and filling-in occurred across them. It suggests that visual surface representations can be formed based on local edges and filling-in process between them, rather than global luminance profiles, or spatial context of visual shapes.

[1]*Beth Israel Deaconess Medical Center, Harvard Medical School, Cambridge, MA, USA*

References

(1) Kamitani, Y. and Shimojo, S. (2002) Vision Sciences Society.
(2) Kamitani, Y. and Shimojo, S. (2001) Vision Sciences Society.

431. Sound-induced illusory visual motion

Ladan Shams, Sam Thompson, Shinsuke Shimojo, John Allman

Vision is considered the dominant modality particularly in the domain of spatial perception. Thus, judgments on the spatial aspects of a visual stimulus are not expected to be significantly affected by characteristics of accompanying sounds, etc. We provide evidence to the contrary.

Methods: A red uniform disk was briefly flashed either in the fovea or in periphery. The flash was accompanied with a brief binaural tone that simulated a sound: a) stationary in space; b) moving horizontally to the left; or c) to the right. Participants were asked to judge in each trial whether the disk was stationary, or moving horizontally. Eye movements were monitored throughout the experiment. Although the visual stimulus was stationary in all trials, observers perceived the disk (only when in periphery) as moving in the majority of trials when it was accompanied by auditory motion. We refer to this phenomenon as sound-induced illusory visual motion. In a second experiment, we added conditions in which the disk was physically moving to the left or right. As before, observers reported perceiving motion when the stationary disk was accompanied by moving sound. In addition, we found a reverse effect: some of the moving flashes that were accompanied by stationary sound were perceived as stationary. This effect was significantly smaller than the illusory motion effect, however. In a third experiment, in which the continuously moving sound was replaced by discrete sounds, we found that illusory visual motion can also be induced by apparent auditory motion.

Conclusion: Various control conditions indicated that the illusory motion is a perceptual illusion and is not due to a cognitive bias derived from knowledge of the sound. The results altogether demonstrate that a moving sound (real or apparent) can induce perception of motion

for a temporally coincident visual stimulus in the periphery. These findings counter the general belief that vision is unconditionally the dominant modality in spatial perception.

Reference

Shams, L., Thompson, S., Allman, J. and Shimojo, S. (2002) Vision Sciences Society.

432. Illusory visual motion induced by sound

Ladan Shams, Sam Thompson, John Allman, Shinsuke Shimojo

Vision is considered the dominant modality particularly in the domain of spatial perception. Thus, judgments on the spatial aspects of a visual stimulus are expected not to be significantly affected by characteristics of accompanying sounds, etc. We tested this hypothesis.

Methods: A white uniform disk was briefly flashed either in the fovea or in periphery. The flash was accompanied with a brief binaural tone that induced the sensation of sound emitted from: a) a stationary source; b) a source moving horizontally to the left; or c) a source moving horizontally to the right. Participants were asked to judge in each trial whether the disk was stationary, moving to the left, or moving to the right.

Results: Despite the fact that the visual stimulus was stationary in all trials, subjects perceived the disk in the periphery as moving in 65% of trials where it was accompanied with auditory motion. We refer to this phenomenon as sound-induced illusory visual motion. In contrast, when the disk appeared in the fovea it was perceived as moving in only 20% of the auditory motion trials. These results suggest that the illusory motion is a perceptual illusion and is not due to a cognitive bias derived from knowledge of the sound. We are currently investigating whether it is the spatial auditory localization or motion that underlies this illusion.

Conclusions: We had previously reported a visual illusion induced by sound [Shams *et al*. (2000) *Nature*]. The illusory visual motion reported here, provides another example of how auditory signals can modify the phenomenological quality of visual perception. Furthermore, this illusion counters the general belief that vision is the dominant modality in spatial perception. It appears that the perception of visual stimuli in the periphery is especially malleable by (or dependent on) the signals of other modalities.

References

(1) Shams, L., Thompson, S., Allman, J. and Shimojo, S. (2002) Vision Sciences Society.
(2) Shams, L., Kamitani, Y. and Shimojo, S. (2000) *Nature* **408**:788-788.

433. Brain mechanisms underlying a sound-induced visual illusion

Sam Thompson, Ladan Shams, Yukiyasu Kamitani[1], Shinsuke Shimojo

When a single flash is accompanied with two auditory beeps, the single flash is perceived as two flashes [Shams *et al*. (2000) *Nature*]. This illusion is much stronger in the periphery than fovea. We investigated whether this cross-modal influence on visual perception occurs at the level of visual pathway. We compared the visual evoked potentials (VEPs) in the presence and absence of sound. We measured the VEPs of 15 participants in six different conditions. Conditions Vp and Vf consisted of visual stimulation: a small disk flashed once at a) 8° eccentricity (periphery) or b) 0° (fovea), respectively. Control condition A consisted of auditory stimulation (two beeps). Conditions AVp and AVf consisted of audio-visual stimulation; corresponding to the combination of the stimuli in conditions A with Vp and Vf, respectively. In another control condition, Vp2, a physical double flash at 8° eccentricity was presented. VEPs were recorded from occipital electrodes. For each electrode, the difference wave AV-(A+V) was calculated for periphery and fovea separately. These were taken as measures of change in the activity of the visual areas by sound. Activity was modulated significantly already prior to 200 ms poststimulus in periphery but not in fovea. In addition, the illusory second flash VEP (or AV-(A+V)) was qualitatively very similar to physical second flash VEP (Vp2-Vp), suggesting that the same mechanism underlies the percept of the illusory flash and a physical flash. These results suggest that the activity in the visual cortex is modulated by sound. This implication challenges the general belief that the visual cortical processing is independent of other modalities.

[1]*Beth Israel Deaconess Medical Center, Harvard Medical School, Cambridge, MA, USA*

References

(1) Thompson, S., Shams, L., Kamitani, Y. and Shimojo, S. (2002) Society for Neuroscience.
(2) Shams, L., Kamitani, Y. and Shimojo, S. (2000) *Nature* **408**:788-788.

434. Visual cortex as a site of cross-modal integration

Ladan Shams, Shigeki Tanaka[1], Geraint Rees[2], Sunao Iwaki[3], Shinsuke Shimojo, Toshio Inui[4]

It has been shown that visual perception can be strongly affected by auditory stimuli. It is unclear, however, what the brain circuitry subserving these interactions may be. We used the sound-induced illusory flash effect (a single flash accompanied by two auditory beeps is perceived as two flashes) as a tool to investigate this question. This illusion is much stronger in the periphery than fovea. In a previous study using event-related brain potentials, we had investigated whether this illusion is due to modulation of activity in the visual pathway or a higher perceptual area, such as associative

cortex. We compared the visual evoked potentials (VEPs) in the presence and absence of sound. Activity was modulated significantly already prior to 200 ms poststimulus in the illusion trials (periphery) but not in trials where no illusion occurred (fovea). In addition, the VEP associated with the illusory second flash was qualitatively very similar to the VEP associated with a physical second flash, suggesting that similar representations underlie the percept of the illusory and a physical flash. These results suggested that the observed modulation of activity by sound occurs within the visual cortex. In the present study we tried to localize the brain regions involved in the perception of the illusory flash more directly and more accurately using event-related fMRI. We collected the functional images of 3 participants in the following conditions. Unimodal conditions V_p and V_f consisted of visual stimulation: a small disk flashed once in the periphery or fovea, respectively. Bimodal conditions AV_p and AV_f consisted of auditory-visual stimulation: combination of 2 beeps with visual stimuli V_p and V_f, respectively. In another unimodal condition, V_p^2, a physical double flash was presented in the periphery. Trials were randomized. Contrasting the (illusion) condition AV_p against V_p resulted in activity in Brodmann's areas 17, 18, and 19. Contrasting (no-illusion) condition AV_f versus V_f, however, did not show any activity in the occipital lobe ruling out the possible role of attention in the aforementioned enhanced visual activity. Considering that the visual stimulus was identical in AV_p and V_p, the enhanced activity of early visual areas in AV_p can only be attributed to the perception of the illusory flash caused by sound. Similar brain areas were indicated when contrasting V_p^2 against V_p. The common brain areas involved in these two contrasts confirm our previous ERP results suggesting similar mechanism underlying the percept of a physical and an illusory flash. These results altogether indicate that the activity in the early visual cortical areas is modulated by sound.

[1]*Jin-ai University, Japan*
[2]*University College London, England*
[3]*National Institute of Advanced Industrial Science and Technology, Japan*
[4]*Kyoto University, Japan*

Reference

Shams, L., Tanaka, S., Rees, G., Iwaki, S., Shimojo, S. and Inui, T. (2002) International Multisensory Research Forum.

435. Sensory modalities are not separate modalities: plasticity and interactions

Shinsuke Shimojo, Ladan Shams

Historically, perception has been viewed as a modular function, with the different sensory modalities operating independently of each other. Recent behavioral and brain imaging studies challenge this view, by suggesting that cross-modal interactions are the rule and not the exception in perception, and that the cortical pathways previously thought to be sensory-specific are modulated by signals from other modalities.

Reference

Shimojo, S. and Shams, L. (2001) *Curr. Op. Neurobiol.* **11**:505-509.

436. Sound alters visual evoked potentials in humans

Ladan Shams, Sam Thompson, Yukiyasu Kamitani[1], Shinsuke Shimojo

When a single flash is accompanied by two auditory beeps, the single flash is perceived as two flashes. We investigated whether this crossmodal influence on visual perception occurs at the level of the modality-specific visual pathway or later. We compared the visual evoked potentials (VEPs) in the presence and absence of sound. Activity was modulated extensively and with short latency in trials in which an illusory flash was perceived. In addition, the brain potentials for the illusory flash were qualitatively very similar to those for a physical flash, suggesting that the same mechanism underlies the percept of both illusory and physical flashes. These results suggest that the activity in the visual cortex can be modulated by sound. This implication challenges the general belief that the visual cortical processing is independent of other modalities.

[1]*Beth Israel Deaconess Medical Center, Harvard Medical School, Cambridge, MA, USA*

Reference

Shams, L., Kamitani, Y., Thompson, S. and Shimojo, S. (2001) *Neuroreport* **12**:3849-3852.

437. Painting by mind's eye: Investigating the patterns of functional integration between cortical regions in artists

Joydeep Bhattacharya, Hellmuth Petsche[1] Shinsuke Shimojo

Although appreciation and spontaneous creation of visual art are intrinsic elements common across cultures and races, little is known about the functioning of the human brain while mentally composing an artwork. In this study, this issue was addressed by analyzing multivariate EEG signals obtained from two broad groups - artists (professionally trained in fine art) and non-artists - while they were mentally drawing paintings of their own choices. EEGs at rest were also considered. Our analysis was motivated by the hypothesis that mentally composing an art, like other complex cognitive act (Bhattacharya *et al*., 2001), requires not only co-activation of distributed cortical regions but also functional interdependences between them. To assess the hidden coupling or synchrony between multiple cortical regions, three measures, inspired by nonlinear dynamical system theory, were applied as follows: (*i*) index based on generalized synchrony (dynamic correlations including asynchronous coupling), (*ii*) mean phase coherence (circular variance of the angular phase difference), and (*iii*) phase synchrony

index based on entropy (based on the generalization of noisy and chaotic coupled system in a common framework); results consistent across all three measures were emphasized. Comparing task (mental drawing) to rest, artists showed extensive delta and theta band synchrony over multiple cortical regions bilaterally and higher beta and gamma band synchrony in temporal cortex, whereas non-artists showed enhancement, to a lesser extent, primarily in frontal regions over multiple frequency bands. Comparing two groups during the task, significantly higher delta band synchrony was found in artists as compared with non-artists, yet desynchronization was found in the alpha band in artists; higher synchrony in low frequency band (primarily delta) is likely due to an involvement of a more advanced long-term visual art memory in artists. The results indicated that the patterns of functional integration during spontaneous mental creation of paintings are significantly different in artists from that in non-artists.

[1]*Austrian Academy of Science, Vienna, Austria Brain Research Institute, Vienna, Austria*

References

(1) Bhattacharya, J., Petsche, H. and Shimojo, S. (2002) Vision Sciences Society.
(2) Bhattacharya, J. and Petsche H. (2002) *Cogn. Brain Res.* **13**:179-186.
(3) Bhattacharya, J. *et al.* (2001) *J. Neurosci.* **21**:6329-6337.

438. A computational model of stereopsis that produces depth from interocularly unpaired points as well as binocular rivalry

Ryusuke Hayashi, Taro Maeda[1], Susumu Tachi[1], Shinsuke Shimojo

Half-occluded zones (visible from only one eye) are found at every depth discontinuity in daily visual scenes. Even though such zones have no counterpart in the other eye (thus no disparity defined), they are perceived at a certain depth behind the occluding surface rather than causing binocular rivalry. Here we propose a mechanism detecting interocularly unpaired zones in each eye modeled after physiological responses of disparity selective cells and show a stereo algorithm that reconstructs 3D structures from not only interocularly paired but also unpaired points. In our model, we assume left and right unpaired point detection cells in addition to depth detection cells. These 3 types of cells cooperatively interact with each other depending on physical constraints (uniqueness, smoothness, occlusion) to estimate depth and determine which zones are unpaired. Moreover, since it is contradictory for *monocularly* visible zones to be visible in *both eyes*, we introduce mutual inhibition between left and right unpaired point detection cells. When input images including unpaired zones satisfy occlusion geometry, the model outputs the depth of the zones. The interesting finding is that when we input two different images to the eyes, the model shows an unstable output that alternates between interpretations of monocularly visible zones for the left and the right eyes, thereby reproducing binocular rivalry. Our results suggest that binocular rivalry is an erroneous output of a stereo mechanism that estimates the depth of half-occluded points. There are two general theories for what the rivals are in binocular rivalry: the two eyes, or representations of two different stimuli. We propose a new hypothesis that bridges these two: *interocular* inhibitions between *representations* of monocularly visible zones cause binocular rivalry. Unlike the traditional interocular theory, the level of the inhibitions here is after binocular convergence, thus open to a stimulus-specific mechanism.

[1]*University of Tokyo, Tokyo, Japan*

Reference

Hayashi, R., Maeda, T., Tachi, S. and Shimojo, S. (2002) Vision Sciences Society.

439. Recovery of visual perception from adaptation by sound: The cross-modal "beating heart" effect

Bhavin R. Sheth, Shinsuke Shimojo

In a variant of Troxlers filling-in, nearly equiluminant, chromatic flickering stimuli fade away quickly. We found that a series of sounds synchronized with such a stimulus aids its recovery, and delays its fading. Two red disks (2.5o dia.) centered 5.2o left and right of fixation were alternately flashed for 17 ms each (187 ms inter-stimulus interval, or ISI) on an equiluminant green background at 2.5 Hz for 3 min. The background filled in perceptually. On trials that followed, a 14.9 s re-adaptation phase was followed by a 6.4 s long pairing of one of the flickers with a 11 dB 2 ms long, spatially non-localized click. Subjects (Ss; n=5) had to pick the more visible or visually salient disk regardless of sound. They chose the one coincident with the sound on 81% (range: 73-90%) of trials. General arousal or eye movements cannot explain the data since they would make both stimuli equally visible. Randomly flickered stimuli (107-267 ms ISI) gave a similar result (n=3; 76%). Next, we asked whether sound can protect the visual stimulus from fading. From the trials outset, one disk was paired with a coincident sound, and Ss (n=5) waited for one of the two disks to fade. On 73% of trials (60-100%), the stimulus not coincident with the sound faded first. The protection by sound was transient though: In the third experiment, the sound was turned off after 24 s, and the two stimuli alternated for 2.1 s thereafter; the stimulus paired earlier with the sound was judged more visible after the sound was turned off on 47% (6 Ss, 27-60%) of trials only. We speculate that attention to a stimulus at some location in visual space is enhanced by a sound synchronized with it. Our findings agree with past reports of audition affecting vision; we go beyond them in showing the role of synchronized sound in visual perception.

Reference

Sheth, B. and Shimojo, S. (2002) Visions Sciences Society.

440. How the lack of visuomotor feedback affects even the early stages of goal-directed pointing movements

Bhavin R. Sheth, Shinsuke Shimojo

Pointing movements made with a hidden cursor from the center of gaze to a stationary, visible target overshot the actual target location. The systematic error decreased when the final cursor location from the previous trial was shown, which likely led to the creation of an internal sensorimotor model of movement. However, the putative model had a short memory, and could not substitute for on-line visuomotor feedback on subsequent trials. Contrary to common belief, the effect of a lack of visuomotor feedback was seen even in the early acceleration stage of the movement trajectory. Unchecked in the absence of visual monitoring, the acceleration stage of the movement lasted longer, as was evidenced by the significantly larger value of the peak cursor speed. Moreover, the speed peaked much later in the course of the movement. Speed declined more rapidly thereafter. Consequently, the delayed deceleration stage lasted far less than the acceleration stage. In the absence of visual feedback, the shift rightward in time of the peak speed position (PSP) in relation to total movement duration and other changes in the trajectory imply that visual feedback must play a significant role in determining when acceleration ceases (dV/dt=0), and argue against the traditional notion that visuomotor feedback is unavailable until the later stages of movement. Moreover, our data suggest that non-visual modalities, e.g., proprioception, may be too slow to make up for the absence of vision.

Reference

Sheth, B. and Shimojo, S. (2002) *Exp. Brain Res.* **143**:181-190.

441. Signal strength determines the nature of the relationship between perception and working memory

Bhavin R. Sheth, Shinsuke Shimojo

Neurophysiological and behavioral studies have shown that perception and memory share neural substrates and functional properties. But are perception and the active working memory of a stimulus one and the same? To address this question in the spatial domain, we compared the percept and the working memory of the position of a target stimulus embedded within a surround of moving dots. Motion in a particular direction after the targets offset biased the memory of target location in the same direction. However, motion simultaneous with a high contrast, perceptually strong target biased the percept of target location in the opposite direction. Thus, perception and working memory can be modified by motion in qualitatively different ways. Manipulations to strengthen the memory trace had no effect on the direction of the memory bias, indicating that memory signal strength can never equal that of the percept of a strong stimulus. However, the percept of a weak stimulus was biased in the direction of motion. Thus, although perception and working memory are not inherently different, they can differ behaviorally depending on the strength of the perceptual signal. Understanding how a changing surround biases neural representations in general, and post-sensory processes in particular, can help one understand past reports of spatial mislocalization.

Reference

Sheth, B. and Shimojo, S. (2002) *J. Cogn. Neurosci.* In press.

442. Dynamic shape integration in extrastriate cortex

Carol Yin, Shinsuke Shimojo, Cassandra Moore[1], Stephen A. Engle[1]

In anorthoscopic viewing conditions, observers can perceive a moving object through a narrow slit even when only portions of its contour are visible at any time. We used fMRI to examine the contribution of early and later visual cortical areas to dynamic shape integration. Observers' success at integrating the shape of the slit-viewed object was manipulated by varying the degree to which the stimulus was dynamically distorted. Line drawings of common objects were either moderately distorted, strongly distorted, or shown undistorted. Phenomenologically, increasing the stimulus distortion made both object shape and motion more difficult to perceive.

We found that bilateral cortical activity in portions of the ventral occipital cortex, corresponding to known object areas within the lateral occipital complex (LOC), was inversely correlated with the degree of stimulus distortion. We found that activity in left MT+ showed a similar pattern as the ventral occipital region. LOC also showed greater activity to a fully visible moving object than to the undistorted slit-viewed object. Area MT+, however, showed more equivalent activity to both the slit-viewed and fully visible moving objects. Thus in early retinotopic cortex, the distorted and undistorted stimuli elicited the same amount of activity. Higher visual areas, however, were correlated with the percept of the coherent object, suggesting that the shape integration is mediated by later visual cortical areas. Motion information from the dorsal stream may project to LOC to produce the shape percept.

[1]*University of California Los Angeles, Los Angeles, California, USA*

Reference

Yin, C., Shimojo, S., Moore, C. and Engle, S. (2002) *Curr. Biol*. In press.

443. Development of crossmodal spatial perception in human infants

Patricia Neil, Christine Chee-Ruiter, Rui Wang, Alice Lin, Benjamin Matthews, Shinsuke Shimojo

Under normal conditions, information about our environment begins with disjointed sensory inputs from different modalities, with their various processing rates and resolution capabilities that are then integrated into a unified percept. One of the major components of interest in this crossmodal integration is spatial perception. Although evidence indicates that infants are capable of perceiving some types of crossmodal spatial relations, it is not yet known what their specific perceptual capabilities are at various developmental stages, the particulars of how the integrative mechanism develops, or its contribution to cognitive development. In this project we examine the visual and auditory responses of human infants from birth through nine months of age. Subjects are seated in a darkened, sound-deadened enclosure and are videotaped while viewing unimodal and bimodal visual and auditory stimuli. Head and eye movement responses are analyzed using frame-b!y-frame digitization provided by the program MediaAnalyzer. Data will be incorporated into a computational model. Our method for the study of the development of crossmodal spatial perception, specifically auditory-visual localization, will provide baseline, auditory-visual spatial perceptual data on normal infants and permit the comparison of infant responses with the wealth of information that currently exists from adult human and primate studies.

444. Gaze modulation of visual aftereffects in color and depth

Dylan Nieman, Ryusuke Hayashi, Richard A. Anderson, Shinsuke Shimojo

The gaze modulation of visual aftereffects remains controversial with few exceptions. Here, we test gaze modulation using color and depth aftereffects. Using methods of constant stimuli to derive psychometric functions we measure the intensity of color and depth aftereffects primarily utilizing three paradigms: 1) Classical Retinotopy (Subjects maintain constant fixation throughout adaptation and testing. Adaptation stimuli are always presented on the fovea while test stimuli are presented at varying retinal positions); 2) Balanced Alternating Adaptation (Similar to Mayhew. At regular intervals during the adaptation period, subjects alternate fixation between two loci with opponent adaptation stimuli. For testing, refixation position varies and the test stimuli are presented at the fovea); and 3) Alternating Fixation Adaptation (Similar to 2, except only one of the alternating fixation loci includes an adaptation stimuli). In both color and depth aftereffects we find strong spatial tuning (1) centered at the location of adaptation with significant effect beyond the retinotopic-adapted region. Alternating fixation (2,3) shows strong gaze dependent aftereffects for both color and depth. Alternating adaptation paradigms provide a qualitatively different means of testing the spatial tuning of aftereffects and may prove a more sensitive measure of gaze modulation. The results provide strong evidence for (a) gaze modulation of aftereffects, (b) generality of the modulation across two visual attributes, and, (c) perceptual correlates of the modulation of neural activity by gaze direction.

Reference

Nieman, D., Hayashi, R., Anderson, R. and Shimojo, S. (2002) Vision Sciences Society.

445. What we see is what we like — Intrinsic link between gaze and preference

Claudiu Simion, Christian Scheier[1], Eiko Shimojo[2], Shinsuke Shimojo

Deciding whether we like someone or something has profound influences on our attitudes and behavior. Not much is known about how we make such decisions, and how it is intrinsically linked to orienting behavior such as gaze.

Our past experiments (VSS 01) revealed a bias in gaze direction when subjects compared two faces for attractiveness (faces in a pair were always matched for baseline attractiveness, to make the task difficult). The preferred face was looked at for longer time with the bias gradually increasing from chance level (50%) to 84% just prior to decision, in a "cascade effect." Thus, a cognitive causal pathway (we see an object more because we like it more) and a perceptual-motor pathway (we like it more because we see it more) seem to coexist and form a feed-forward loop, which is necessary before to make a conscious preference decision.

The present study examines robustness and generality of the "cascade effect" in several different directions. When subjects were asked to (a) compare faces that were significantly different (unlike in the previous experiment) in base attractiveness, the bias was still present. Moreover, we revealed it even when subjects compared (b) slightly modified versions of the same face, or when (c) the stimuli to be compared were abstract shapes (Fourier-descriptor generated figures).

The gaze bias curves all fitted on 4 parameter sigmoids, consistent with our model. The saturation levels of the curves correlate with the difficulty of the task, as if the harder the decision, the more "needed" are such biases. We therefore expand our model in light of the new findings, adding a mathematical base to it.

[1]*MediaAnalyzer, Hamburg, Germany*
[2]*Bunkyo Women's University, Hiroshima, Japan*

Reference

Simion, C., Scheier, C., Shimojo, E. and Shimojo, S. (2002) Vision Sciences Society.

446. TMS reveals the correct location of flashes in motion-mislocalization illusions

Daw-An Wu, Shinsuke Shimojo

Transcranial Magnetic Stimulation (TMS) is a safe, non-invasive electrophysiological method of transiently altering brain activity. Dual-pulse TMS to the occipital cortex causes a brief illusory flash (phosphene). Here we show that triggering a phosphene during the viewing of visual stimuli can cause nascent visual cortex activity to be revealed to consciousness. First we trigger phosphenes during a moving visual stimulus. This causes subjects to see an image from the past. They see a sudden repetition of the stimulus as it was earlier ("retrieval"). Second, this effect is used to "retrieve" a flash that had been affected by a mislocalization illusion. In this case, the retrieved flash is not seen as a repetition of the erroneous percept, but is seen at another position reflecting the correct position of the flash.

Experiment 1: TMS is administered while a clock hand rotates around a fixation point. What subjects perceive is the sudden and momentary appearance of another clock hand at the same time as they see the phosphene. This additional hand appears at a location farther back in the clock hand's trajectory. Here, TMS has "retrieved" an image of the clock hand from an earlier point in its trajectory.

Experiment 2a: At some position in the clock's movement, the hand is displayed with a different color ("flash"). TMS is not used. Viewers do not detect discontinuity, but report the position of the flash erroneously, biased further forward in the trajectory. Displacements cross both meridianal borders.

Experiment 2b: TMS is administered with the visual stimulus of 2a, usually ~300ms after the flash. Because TMS is delayed, subjects first see the flash as in 2a, coming before the phosphene. At the time of the phosphene, a second color flash is seen. Subjects' reports of its location reflect the actual location of the flash. Here TMS has "retrieved" an image that was not previously seen, and uncovers a nascent, more accurate representation of the color flash's position.

Experiment 3a: The clock's direction reverses at the flash. TMS is not used. Subjects' perceptions are qualitatively correct: the hand is seen to flash and bounce back at the same time. However, their reports of the flash's location reveal that they see a blunted version of the motion sweep. Reports of the flash's location fall short of the true position by as much as 20 degrees.

Experiment 3b: TMS is administered with the visual stimulus of 3a. The same effect is found as in 2b. Additionally, the perceptual blankness around the true flash position makes observation clearer at shorter TMS delays, revealing the clearest "retrieval" with TMS at ~200ms after the flash.

Discussion: In these experiments, TMS makes neural excitability states literally visible, revealing moments in a dynamic cortical process. This may reflect an interaction with residual neural activity following visual stimuli. The optimal position of the stimulating coil in 2b and 3b suggests that during the viewing of the above illusions, a correct representation of the color flash is linked to lower visual cortex, but this representation does not normally reach consciousness intact.

Reference

Wu, D-A. and Shimojo, S. (2002) Vision Sciences Society.

Publications

Bhattacharya, J. and Petsche H. (2002) Shadows of artistry: Cortical synchrony during perception and imagery of visual art. *Cogn. Brain Res.* **13**:179-186.

Bhattacharya, J., Shams, L. and Shimojo, S. Critical role of gamma band responses in the sound induced illusory double flash perception. *NeuroReport*. In press.

Bush, E.C., Shimojo, S. and Allman, J.M. (2002) Rapid long lasting learning in a collinear edge detection task. *Perception* **31**:791-798.

Shams L, *et al*. (year-?) Sound alters visual evoked potentials in humans. *Neuroreport* **12**:3849-3852.

Shams, L., Shimojo, S. and Kamitani, Y. (2001) A visual illusion induced by sound. *Cogn. Brain Res.* **14**:147-152.

Sheth, B.R. and Shimojo, S. (2002) How the lack of visuomotor feedback affects even the early stages of goal-directed pointing movements. *Exp. Brain Research* **143**:181-190.

Sheth, B. and Shimojo, S. (2001) Compression of space in visual memory. *Vision. Res.* **41**:329-341.

Sheth, B. and Shimojo, S. Signal strength determines the nature of the relationship between perception and working memory. *J. Cogn. Neurosci.* In press.

Shimojo, S. (2002) A new type of afterimage based on cortical representation of visual surfaces. *Brain Science*, vol. 24, no. 2.

Shimojo, S., Kamitani, Y. and Nishida, S. (2001) Afterimage of perceptually filled-in surface. *Science* **293**:1677-1680.

Shimojo, S., Scheier, C.R., Nijhawan, R., Shams, L., Kamitani, Y. and Watanabe, K. (2001) Beyond perceptual modality: auditory effects on visual perception. *The Journal of the Acoustical Society of Japan* (E), 22 (2), 200 (E), 22 (2), (English edition). The Journal of the Acoustical Society of Japan, 57, (3) (Japanese edition).

Shimojo, S. and Shams, L. (2001) Sensory modalities are not separate modalities: plasticity and interactions. *Curr. Op. Neurobiol.* v. 11, no. 4, 505-509.

Taga G, *et al*. (2002) Visual feature binding in early infancy. *Perception* **31**:273-286.

Watanabe, K., Nijhawan, R. and Shimojo, S. (2001) *J. Exp. Psychol. Human* **27**:879-894.

Watanabe, K., Imada, T., Nihei, K. and Shimojo, S. Neuromagnetic responses to chromatic flicker: Implications for photosensitivity. *Neuroreport*. In press.

Yin, C., Shimojo, S., Moore, C., Engle, S. (2002) Dynamic shape integration in extrastriate cortex. *Curr. Biol.* In press.

Facilities

Biopolymer Synthesis Center
Flow Cytometry/Cell Sorting
Genetically Altered Mouse Production Facility
Monoclonal Antibody Facility
Nucleic Acid and Protein Sequence Analysis Computing Facility
Protein Expression Center
Protein Microanalytical Laboratory

Biopolymer Synthesis Center

Supervisor: Suzanna J. Horvath
Staff: Andrej A. Ausing, Christine E. Lisnock, John Racs, Ryan C. Thomson, Ann P. Vitti

The Biopolymer Synthesis Center has continued to provide high quality chemical synthesis, purification, and analysis of a wide range of biopolymers at the request of the scientific community at Caltech. We also provide strong technical support to individual research projects through the introduction and the use of the latest technology in both chemistry and biology. Today's emerging new techniques challenge us with their sophistication and complexity, as well as their rapid space of evolution. For a growing number of scientists we have made complex experiments possible by advance design, synthesis, and purification of unique molecules. The intellectual interaction between our staff and the research scientist was essential to the success of each project.

The DNA/RNA Synthesis Laboratory

The total number of DNA molecules synthesized in FY'01 is 12,939 for 53 Caltech faculty members and three outside collaborators, including JPL. This represents 397,751 base additions.

The total number of RNA molecules synthesized in FY'01 was 118 for nine Caltech faculty members. This represents 2,711 base additions.

Our primary instruments are two ABI Model:3948 synthesizers. Each instrument is able to synthesize 48 purified deoxyoligonucleotides up to 91 bases in length every 36 hours. The synthesis scale is 0.05 μM.

The three four-column DNA/RNA synthesizers (ABI Model:394-08) operate with full capacity all the time. These synthesizers can be set up as 0.04, 0.2, or 1.0 μM scale synthesis. They are flexible for the use of a variety of modified chemistries, including RNA synthesis. A catalog of DNA reagents for chemical modifications is available in our laboratory. With the expert assistance of our DNA synthesis staff, Caltech scientists can choose from this catalog and design "special oligos" for synthesis. These special oligos are now widely used at Caltech in a large variety of experiments.

Requests for chemically-modified oligonucleotides have been increasing dramatically and continuously and they are more challenging than ever before.

The Peptide Synthesis Laboratory

There are four automated peptide synthesizers in our Peptide Synthesis Laboratory. Our most advanced solid-phase automated synthesizers are the two ABI Model:433A. They are set up for the application of Fmoc chemistry with optimized chemical cycles. These cycles are automatically monitored by a conductivity cell. Using Fmoc chemistry, we can selectively introduce phosphorylated sites on any Ser, Thr, or Tyr residues in the peptide sequence. There are two automated peptide synthesizers, ABI Model:430A, set up for the application of t-Boc chemistry. We have synthesized large numbers of short peptides, peptide-protein conjugates to generate heterogeneous antisera or monoclonal antibodies with desired specificity for a large variety of applications. Each peptide made in our laboratory includes CE, HPLC, and MS analysis. MS analysis is usually done at the Protein/Peptide Microanalytical Laboratory (PPMAL).

The number of peptide molecules synthesized so far in FY'02 remained high, with a large increase in their complexity and purity requirements.

In FY'01 we synthesized 196 peptides for 27 Caltech faculty members and nine outside collaborators. This represents a total of 2,296 regular and 185 special amino acid couplings.

A series of technically difficult peptide synthesis were carried out successfully in our laboratory that contributed to the substantial progress made in the laboratories of the following professors: David Baltimore (Biology); William Dunphy (Biology); Thomas Meade (Biology); Alexander Varshavsky (Biology); Peter Dervan (Chemistry); and David Tirrel (Chemistry).

We have synthesized a large number of extremely difficult, very insoluble hydrophobic peptides for Dr. Boris Minev and Professor Maurizio Zanetti at the UCSD Cancer Center.

For Professor David Eisenberg's request, we made a series of peptides with exceptional high purity for his crystallography studies. He is at the UCLA-DOE Laboratory of Structural Biology, Molecular Medicine.

For the fifth year in a row, in collaboration with Professor Michael Grunstein at the Molecular Biology Institute, UCLA, we have been synthesizing a large number of histone-peptides that involves the combined application of both t-Boc and Fmoc synthesis. We have found a way to introduce selective acetylation of one or more Lys side chains in a given peptide sequence.

Flow Cytometry Cell Sorting Facility

Supervisor: Ellen Rothenberg
Member of the Professional Staff: Rochelle A. Diamond
Staff: Patrick F. Koen

The Caltech Flow Cytometry Cell Sorting Facility provides the Caltech Community with essential technical expertise and access to equipment for flow cytometric analysis and cell purification by sorting on a fee-for-service basis. Investigators consult with the facility staff on their particular experimental plans in order to optimize their samples for existing standard protocols or to construct custom protocols designed for their particular needs.

Flow cytometry is a method for quantitating components, structural features, or functions of cells

primarily by optical means. Flow cytometers involve sophisticated fluidics, laser optics, electronic detectors, analog to digital converters, and computers. The optics deliver laser light focused to a beam a few cell diameters across. The fluidics hydrodynamically focus the cell stream to within a small fraction of a cell diameter, and, in sorters, break the stream into uniform-sized droplets to separate individual cells. The electronics quantitate the flashes of scattered and fluorescent light, and, under computer control, electrically charge droplets containing cells of interest so that they can be deflected into a separate test tubes or culture wells. The computer records data for thousands of cells per sample, and displays the data graphically. Essentially any cell or cell-like particle can be analyzed and isolated from a heterogeneous population based on its scatter and fluorescent properties. This allows for qualitative as well as quantitative information on the levels of a given molecule on or inside individual cells which are then correlated to reveal statistical population dynamics for all the cells in the sample. Although one cell at a time is measured, thousands of cells can by analyzed and sorted in just a few seconds. Cells may be alive or fixed at the time of measurement as long as they are monodispersed (in a single cell suspension). This technology has many applications and is driven by the needs of its users. Some of the applications for cell biology include isolation of transfectants exhibiting fluorescent reporter genes; phenotyping and isolation of cell populations; cell cycle analysis including newly synthesized DNA (by BrdU uptake); real-time measurements of cellular physiological responses such as intracellular free calcium, membrane potential, pH, cell death quantitation; and isolation of clones for high-throughput genomics.

The Facility offers cell sorting and analysis on two full-service, dual-laser cell sorters (Becton-Dickinson FACS Vantage SE and Coulter Epics Elite as well as round-the-clock access to a nonsorting dual-laser flow cytometric analyzer (Becton-Dickinson FACSCalibur). Scheduling is permitted in advance for up to a month, to enable researchers to plan experiments with time-dependent samples. A special aspect of this Facility is the extensive consultation, advice on trouble-shooting, and support for custom applications that is provided to any current or prospective user interested in the use of this technology. More information about the Facility can be obtained at its web site: http://www.its.caltech.edu/~cellsort.

This past year the facility has serviced 46 individual users representing 12 on-campus groups. The following are highlights from various on-going projects of those investigators.

David Anderson Group

Yosuke Mukouyama and Donghun Shin are investigating the development of blood vessels from distinct subsets of embryonic precursor cells. They have found that early angiogenic cells in embryonic yolk sac are already separated into arterial and venous types from the first stage of vessel formation. They have used FACS to target cells with LacZ using live intracellular FACS-GAL staining in conjunction with surface staining to sort the marked arterial or venous precursors form appropriate knock-in mice. These cells can then be tested for differential expression specific genes of interest using RT-PCR. To investigate the innate or interaction-dependent origins of the distinct arterial and venous sets of endothelial cells, primitive yolk sac hemangioblasts are purified by sorting and allowed to differentiate in culture. In these experiments, before ephrinB2 expression turns on, cells expressing the very early hemangiogenic marker Flk-1 are sorted from embryonic yolk sac and plated in clonal culture to determine the frequency of clones that eventually turn on the arterial marker ephrinB2. In a complementary set of approaches, they are attempting to determine whether any subset of hemangiogenic stem cells may persist at any later developmental times. Flk-1^+ adult cells which do not express the mature endothelial marker PECAM (mostly thought to be hematopoietic stem cells) are purified by sorting and tested in angiogenic cultures *in vitro* to determine whether any of them retain angiogenic activity.

A long-standing question is how vessel formation is guided, and developing peripheral nerves are a possible source of organizing signals. Blood vessels develop in close proximity to nerves, and it is known that the same ephrinB2/EphB signaling system that organizes arterial and venous endothelial structures can also direct nerve guidance. Therefore, we are sorting fetal endothelial precursors ($PECAM^+$) and combining them with neuronal or glial cells, sorted on the basis of $p75^{trkA}$, NCAM, Neurotag or P_0 expression, so that their interactions can be investigated directly in coculture *in vitro*. Preliminary results indicate that crosstalk may occur in these conditions. By isolating the $lacZ^+$ endothelial precursors from the ephrinB2 or EphB4 knock-in mice, it is possible to determine the contributions of arterial vs. venous cells to this interaction.

Christian Hochstim is working with oligodendrocytes, the primary myelinating glial cell of the central nervous system, which are generated from a small ventral domain of the ventricular zone of mouse spinal cord in late embryogenesis (E12-E18). These precursors migrate and differentiate to populate diffuse areas of gray and white matter. Various surface markers such as NG2, O4, PNA-lectin and PDGFRa are expressed by precursors but not mature oligodendrocytes. Most oligodendrocyte precursors are thought to be specified in a domain known as pMN, defined by the expression of the bHLH transcription factor Olig2. In chick spinal cord, oligodendrocyte precursors are also specified from the more ventral p3 domain and express Nkx2.2 but not Olig2. It is not clear whether or not there are also two populations of oligodendrocyte precursors in the mouse or what if any differences in developmental potential exist between these different progenitors. Using mice transgenic for a GFP knock-in at the Olig2 locus, they dissociate, stain and sort cells from embryonic spinal cords at the stages of

oligodendrocyte generation (E14.5-16.5). In these mice, precursors positive for Olig2 expression will be GFP+. Fluorescent antibody staining for oligodendrocyte precursor markers (PE and/or APC conjugated) is being used in combination with GFP/Olig2 expression to prospectively identify and isolate by flow cytometry cells which are oligodendrocyte precursor antigen positive and GFP positive, and cells which express precursor antigens but are GFP negative. These two populations will then be analyzed under various culture conditions to compare their developmental potential/plasticity and properties of migration to try to identify any differences between different subpopulations of oligodendrocyte progenitors.

The aim of Limor Gabay's research is to test the specificity of differentiation potential in CNS stem cells derived from different positions along the dorsal-ventral axis of the spinal cord. She is checking whether growing the stem cells *in vitro* in a neurosphere culture changes their properties by isolating the cells from the embryonic mouse spinal cord. During embryonic development the spinal cord cells are differentiated in response to two inductive signals: one of them is Shh, which comes from the floor plate, and the other is TGF-b, which comes from the roof plate. As a result the spinal cord is differentiated into distinct domains marked by gene expression, each giving rise to different subtypes of neurons and glia. Using FACS she isolating two groups of progenitors, one from the dorsal and one from the ventral part of e14 mouse spinal cord. In the ventral part she is selecting for a specific type of progenitor that has been characterized in the Anderson lab. These cells are marked by the expression of the Olig2 gene and can give rise to motoneurons and oligodendrocytes. Using transgenic mice, which express the GFP gene under the control of the Olig2 promoter, which enables the isolation of the cells by FACS (Zhou and Anderson, 2002). The cells from the dorsal part of the spinal cord do not express Olig2 and give rise *in vivo* to different subtypes of neurons and also astrocytes, but not to oligodendrocytes and motoneurons. The properties of the sorted cells can be checked after growing them *in vivo* in a neurospheres culture.

Kenji Orimoto is currently trying to identify potential genes involved in neural differentiation of stem cells and early markers for Schwann cell lineage by cDNA subtraction with freshly isolated sciatic nerve cells using cell surface markers and FACS. Several candidate genes were identified and further studies are in progress. Furthermore, they have demonstrated that neural crest stem cells can undergo cell-intrinsic changes in their sensitivity to instructive signals, while maintaining multipotency and self-renewal capacity (Kubu *et al*., 2002). Taken together, FACS system enables us to study the mechanisms underlying the cell fate determination of neural stem cell at molecular and cellular level.

Judith L. Campbell group

Isabelle Lesur has been using gene expression profiling to identify pathways involved in the premature aging of *dna2-1*, a *Saccharomyces cerevisiae* DNA replication mutant. The DNA content was analyzed by the propidium iodide/RNAse flow cytometric protocol in old and young *dna2-1* cells isolated by a centrifugal elutriation.

Jose Alberola-Ila group

The Alberola-Ila group is examining the role of Ras signaling during T-cell development. To determine the expression pattern of various genes involved in this process, they have employed sorting to collect subpopulations of T cells at various stages during their development. In addition, they have used flow cytometry analysis to examine the effects of over-expression of various molecules during T-cell development. Micheline Laurent has been overexpressing a Ras pathway inhibitor by retroviral transduction of precursors and using multicolor flow cytometry to analyze the impact on T-cell repertoire selection.

Eric Tse of the Alberola-Ila lab investigates the role of the early growth response (EGR) family of transcription factors in mediating the signals for the positive selection of developing T cells. Expression of dominant negative forms of EGR in transgenic mice, in model cell lines, and in fetal thymic organ cultures (FTOCs) disrupts EGR function, and the phenotyping of this disruption involves extensive use of the FACSCalibur for four-color immunofluorescent analysis. Detection of fluorochrome-conjugated antibodies specific for various extracellular and intracellular proteins provides invaluable information on individual cells' developmental state. This work has also used fluorescence detection to restrict analysis to infected cell subsets expressing dominant negative proteins by virtue of a retroviral expression construct which confers GFP fluorescence, and staining of DNA with propidium iodide has allowed me to look at cell cycle kinetics. Recently, he has also begun to use the sorting facility to sort infected cell populations by GFP fluorescence for subsequent analysis by Western blot.

Gabriela Hernández-Hoyos is studying the role of the Zn finger transcription factor GATA-3, in a project initiated when she worked in the Rothenberg group. GATA-3 is a transcription factor that is essential at least for early T-cell development; this project is to determine what role GATA-3 plays at later stages of development. The overall approach has been to overexpress wild-type GATA-3, a mutant form of GATA-3, or an inhibitor of GATA-3 function, in fetal and adult thymocytes using retroviral vectors, and then analyze changes in their development in fetal thymic organ culture, as well as changes in their patterns of gene expression. To analyze changes in gene expression, thymocytes are transduced with bicistronic retroviral constructs co-expressing GATA-3 (or the other genes) along with GFP. The transduced cells are then sorted away from the non-transduced populations on the basis of GFP fluorescence. In collaboration with Michele K. Anderson of the Rothenberg group, expression of a number of genes has then been analyzed in these populations by quantitative RT-PCR to determine if GATA-3, mutant GATA-3 or the

inhibitor of GATA-3 modify the pattern of expression seen in GFP-only transduced populations.

Richard Roberts group

Shelley Stark in Rich Roberts' lab in Chemistry is detecting protein synthesis in live cells using puromycin conjugates. She is monitoring the amount of puromycin conjugate (F2P) taken up by the cells using flow cytometry. The permeability profile for various puromycin conjugates (B2P, B2P-Me, F2P, F2P-Me, DMT-F2P-Me, Cy3-2P, etc.) in a D9 thymocyte cell line was determined by using a live gate (FSC vs. SSC) and looking a fluorescein fluorescence. The results demonstrate that various conjugates may be used as indicators of protein synthesis in live cells.

Ellen Rothenberg group

The Rothenberg lab depends on flow cytometry in almost every aspect of their research on the regulatory functions that guide hematopoietic precursors to undergo T-lineage commitment. Four-color analysis and occasionally five-color analysis are used to analyze the effects of mutation or misexpression of a variety of regulatory genes in the immature cells. A GFP reporter has been used to monitor in activity *in vivo* of an IL-2 regulatory sequence transgene, which was found to contain a potential locus-activating function. This regulatory region is now being dissected at the molecular level to determine the sites of relevant protein-DNA interactions. In studies involving perturbation of lymphocyte development by retrovirally transduced genes, cells expressing the perturbing function are isolated for mRNA expression analysis and developmental potential tests on the basis of the GFP coexpressed from the retroviral vector. Flow cytometry, with its ability to determine changes in an entire constellation of properties on an individual-cell basis, has become the group's indispensable tool for analyzing the component processes within lineage commitment.

Publications

Anderson, M.K., Hernandez-Hoyos, G., Dionne, C.J., Arias, A.M., Chen, D. and Rothenberg, E.V. (2002) Definition of regulatory network elements for T-cell development by perturbation analysis with PU.1 and GATA-3. *Dev. Biol.* **246**:103-121.

Anderson, M.K., Weiss, A.H., Hernandez-Hoyos, G., Dionne, C.J. and Rothenberg, E.V. (2002) Constitutive expression of PU.1 in fetal hematopoietic progenitors blocks T-cell development at the pro-T cell stage. *Immunity* **16**:285-296.

Kubu, C.J., Orimoto, K., Morrison, S., Weinmaster, G., Anderson, D.J. and Verdi, J.M. (2002) Developmental change in notch1 and numb expression mediated by local-cell interactions underlie progressively increasing delta sensitivity in neural crest system cells. *Dev. Biol.* **244**:199-214.

Mukouyama, Y.-s., Shin, D., Britsch, S., Taniguchi, M. and Anderson, D.J. (2002) Sensory nerves determine the pattern of arterial differentiation and blood vessel branching in the skin. *Cell* **109**:693-705.

Yang, L., Qin, X.-F., Baltimore, D. and Van Parijs, L. (2002) Generation of functional antigen-specific T cells in defined genetic backgrounds by retrovirus-mediated expression of TCR cDNAs in hematopoietic precursor cells. *Proc. Natl. Acad. Sci. USA* **99**:6204-6209.

Genetically Altered Mouse Production Facility

Director: Shirley Pease F.I.A.T.

Mouse Facility Manager: Bruce Kennedy, M.S., RLATG

Mouse Facility Supervisor: (vacant)

Embryonic Stem Cell Culture: Jue Jade Wang, M.S.

Cryopreservation and Microinjection: Juan Silva, B.S., LAT

Staff: Jennifer Alex, ALAT; Armando Amaya; Lilia Anonuevo, ALAT; Cirila Artega; Jenny Arvizu; Donaldo Campos; Hernan Granados; Carlos Hernandez; Stephanie Huang, B.S.; Myka Kairs; Joon Kang; Jorge Mata, ALAT; Jose Mata; Gustavo Munoz, B.A.; Lorena Sandoval; Janet Verduzco; Shannan Witherow, A.A.; Caroline Young, B.S.

The transgenic technique for gene addition (Gordon *et al*., 1980) and targeted gene modification (Zijlstra *et al*., 1989) have been established at Caltech since 1984 and 1993, respectively. Gene addition in the mammalian system is accomplished by injecting DNA into the pronucleus of a fertilized egg. 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. 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, so as to produce descendants that are entirely transgenic, resulting from the ES cell contribution to the germline of the chimeric mouse. More recently, 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, C. *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.

The newly refurbished, pathogen-free barrier-operated mouse facility was opened and re-stocked with pathogen-free strains in February 1995. In addition to 76

transgenic, knockout and knockin 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 maintain 126 separate strains of mouse. Facility staff provides a complete service to investigators. All colony management operations are carried out by staff at the request of investigators. The work of the staff and manager of the facility continues to reflect Caltech's commitment to good laboratory animal practice and our adherence to NIH guidelines. In the past year, the Facility participated in the campus effort to become accredited by the International Association for the Accreditation of Laboratory Animal Care.

The facility, in collaboration with Anderson, Simon, Wold and Varshavsky laboratories, has generated multiple transgenic, knockout and knockin mouse strains. Presently, thirteen principal investigators and their post-doctoral fellows or graduate students use the facility.

New transgenic lines have continually been produced and in all, 55 new transgenic lines for gene addition and 40 new knockouts have been produced since February 1995. This year, 28 transient mouse models for analysis have been generated by use of the lentivirus vector method, together with one Tg rat model. Facility staff has performed all embryo manipulation involved in the production of these new lines. Microinjection equipment has been set up within the barrier facility, which operates on restricted access as part of the "barrier" itself. A room outside the facility has been allocated by the Division to be used primarily for teaching grad students, technicians and postdocs the techniques involved in transgenic mouse production. This room has been operating since July 1996. Investigators have the option of using this room to perform their own microinjection of embryos, rather than using the full technical service available from the Genetically Altered Mouse Facility.

In tissue culture and the use of embryonic stem cells, the Facility participated in the derivation of new ES cell lines derived from genetically altered mice (see Simon laboratory Annual Report, 2001). Goals for the coming year for this part of the Facility include the acquisition and use of hybrid ES cell lines for their reported vigor, and also, the establishment of the tetraploid embryo complementation technique for the generation of animals wholly of ES cell origin.

In the generation of mouse models involving the use of embryonic stem cells, we continue to pre-screen candidate clones for injection by the preparation of chromosome spreads. In establishing the chromosome count of each clone prior to injection, we find we are reliably able to predict which clones will generate good chimeras that will transmit through the germline. In effect, we have been able to increase efficiency by 100% in the production of germline transmitting chimeras.

In cryopreservation, 41 strains are either stored or partially stored. For each strain, between 200 and 500 embryos at 8-cell stage have been preserved in liquid nitrogen. There are currently over 12,000 embryos frozen in total. We shall continue to preserve embryos from every single mouse strain the Core maintains. 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.

In addition to producing a total of 95 new animal models since February 1995, we have re-derived 48 pre-existing genetically altered strains of mice. We continue to re-derive unique strains coming in from other institutions, in order that we might maintain a high standard of pathogen-free animals throughout our facilities.

The production of pathogen-free animals enables Caltech to exchange valuable genetically-altered mouse models with other academic groups around the world. As more and more mouse models become available and the exchange of animals more frequent, it is essential for Caltech to be in a position to deal effectively with animal lines generated in the facility and in other laboratories. To this end, the Division has funded the refurbishment of two quarantine animal holding areas. This now enables us to safely contain and "clean up" other mouse models coming in from other institutions without putting our own colony at risk.

Listed below are the names of the thirteen principal investigators and their postdoctoral fellows or graduate students who are presently using the transgenic facility.

Pepe Alberola-Ila
Susannah Barbee, Christie Beel, Harry Green, Gabriela Hernandez-Hoyos, Micheline Laurent, Eric Tse

David Anderson
Gloria Choi, Xinzhong Dong, Limor Gabay, C.J. Han, Christian Hochstim, Jae Kim, Walter Lerchner, Li Ching Lo, Sally Lowell, Raymond Mongeau, Yosuke Mukoyama, Donghun Shin, Hai Wang, Qiao Zhou, Mariela Zirlinger, Mark Zylka

David Baltimore
Eric Brown, Alexander Hoffman, Carlos Lois, Wange Lu, Mollie Meffert, Lili Yang, Xiao-Feng Qin

Ray Deshaies
Kathy Sakamoto

Scott Fraser
Mary Dickinson, Russ Jacobs, Angelique Louie, Carol Readhead, Seth Ruffins, Chris Waters

Mary Kennedy
Jenia Khoroseva, Irene Knuesel, Pasquale Manzerra, Leslie Schenker

Henry Lester
Chi Sung Chiu, Purnima Deshpande, Carlos Fonck, Cesar Labarca, Raad Nashmi, Clemente Neusch, Johannes Schwarz
Paul Patterson
Zhu Min, Rasika Sowmyalaski
Ellen Rothenberg
Angela Weiss, Mary Yui
Erin Schuman
Changan Jiang
Melvin Simon
Pam Eversole-Cire, Lingjie Gu, Jong-Ik Hwang, Valeria Mancino, Kum Joo Shin
Alexander Varshavsky
Jee Young An, Yong Tae Kwon, Jao Wha Seo, Jun Sheng, Takafumi Tasaki
Barbara Wold
Libera Berghella, Brian Williams

References

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H. (1980) *Proc. Natl. Acad. Sci. USA* **77**:7380-7384.

Liu, X., Wu, H., Loring, J., Hormudzi, S., Disteche, C.M., Bornstien, P. and Jaenisch, R. (1997) *Dev. Dynam.* **209**:85-91.

Overbeek, P.A., Aguilar-Cordova, E., Hanten, G., Schaffner, D.L., Patel, P., Lebovitz, R.M. and Lieberman, M.W. (1991) *Transgenic Res.* **1**:31-37.

Zijlstra, M., Li, E., Sajjadi, F., Subramani, K.S. and Jaenisch, R. (1989) *Nature* (London) **342**:425-638.

Publication

Lois, C., Hong, E.J., Pease, S., Brown, E.J. and Baltimore, D. (2002) Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**:868-872.

Monoclonal Antibody Facility
Supervisor: Paul H. Patterson
Director: Susan Ker-hwa Ou
Staff: Shi-Ying Kou

The Monoclonal Antibody Facility provides assistance to researchers wishing to generate monoclonal antibodies (Mabs), polyclonal ascites Abs (Immunizing the mice with antigen until the serum titer is high enough then induce the mice with sarcoma cells to obtain high titer polyclonal ascites. This method can provide 10-18 ml polyclonal ascites per mouse by using small amount of antigen), ascites fluid or other tissue culture services. In addition to these service functions, the Facility also conducts research on the development of novel immunological techniques.

In collaboration with Li-Ching Lo from Dr. Anderson's laboratory, we tried to compare the footpad immunization protocol with our standard immunization protocol to test and see whether the footpad immunization method can result in production of IgG Mabs with a very short immunization period and produce antibodies with high affinity.

For the footpad immunization protocol, groups of five mice were immunized. 25 μg Ngn-1 in a volume of 25 μl per injection site was applied. Mice were immunized on days-6,-3,0,3,7,10,14 on the right footpad. The left footpad was immunized on days 0,3,7,10,14. On day 15, sacrifice the mice and remove popliteal lymph nodes and spleens separately and proceed with normal fusion protocol.

For the standard immunization protocol, group of three mice were immunized with 50 μg Ngn-1 on day 0, 15, 29, 43, 57, 71. On day 74, sacrifice the mice with the highest titer and remove spleen and proceed with normal fusion protocol.

We obtained Mabs that stain chicken embryo fibroblast cells transfected with Ngn-1, but cannot stain endogenous Ngn-1 on section by either footpad or standard immunizations. The footpad immunization protocol did result in production of IgG Mabs in a 15-day immunization period in comparison to 74 days by standard immunization protocol.

In collaboration with Dr. Kennedy's laboratory, we compared a new adjuvant ImmuEasy with the RIBI adjuvant that we have always used. ImmuEasy adjuvant contains Cp DNA- short pieces of DNA that contain unmethylated cytosine-guanine dinucleotide within a certain base context. Exposure to CpG DNA results in very rapid and strong immune activation. RIBI adjuvant contains TDM (Trehalose dimycolate) and MPL (Monophosphoryl lipid A), TDM is a mycobacterial glycolipid and MPL is a detoxified derivative of bacterial endotoxin. Together they can enhance immune response in mice. After two immunizations with either RIBI adjuvant or ImmuEasy added to the same antigen, we do not see significant difference in the serum titer among the mice. And two out of the six mice that used ImmuEasy formed solid tumor.

In its service capacity, the Facility produced Mabs for the following groups during the past year. Anthony West from Dr. Bjorkman's laboratory obtained Mabs against N-terminal domain of *methuselah* gene from *Drosophila*, Methuselah mutant can extend the average lifespan of *Drosophila* by 30%. Cynthia Chen from Dr. Parker's laboratory obtained Mabs against *Drosophila* RNA Polymerase II C terminal domain. Kennedy laboratory obtained polyclonal ascites against peptide sequence from NR1 and NR2 subunits from the NMDA-type glutamate receptor in the central nervous system. Chiranjib Dasgupta from Dr. Schuman's laboratory obtained polyclonal ascites against rat PUF-part of the *pumilio* gene whose product is known to regulate translation. Liz Haswell from Dr. Meyerowitz laboratory obtained polyclonal ascites against STM120-6His fusion

protein. Joel Pomerantz from Dr. Baltimore laboratory obtained polyclonal ascites against GCC1-gene from mouse thymus.

For the non-Caltech groups, Dr. Wen Shi from UCLA obtained Mabs against Cariogenic Bacteira Candida Albicans, Dr. Kyung Han from Penn State University obtained polyclonal ascites against *Drosophila* fusion protein, Dr. Brian O'Nuallain from University of Tennessee obtained mAbs against different lengths of Huntingtin repeats that is cultured at 370°C, which formed broad ribbon structure and the repeats that is cultured at –200°C and formed thin filaments.

We are currently working with Wange Lu from Dr. Baltimore's laboratory, Qiao Zhou from Dr. Anderson's laboratory, Dr. Strauss' laboratory, Dr. Bjorkman's laboratory, Dr. Parker's laboratory, Rachel Papan from Dr. Zinn's laboratory, Dr. Witte from UCLA, and Dr. Kaufman from UCLA.

Publications

Akutagawa, E. and Konishi, M. (2001) A monoclonal antibody specific to a song system nuclear antigen in estrildine finches. *Neuron* **31**:545-556.

Dubey, P., Wu, H., Reiter, R.E. and Witte, O.N. (2001) Alternative pathway to prostate carcinoma activate prostate stem cell antigen expression. *Cancer Res.* **61**:3256-3261.

Gu, Z., Thomas, G., Yamashiro, J., Shintaku, I.P., Raitano, A., Witte, O.N., Said, J.W., Loda, M. and Reiter, R.E. (2000) Prostate stem cell antigen (PSCA) expression increases with high gleason score, advanced stage and bone metastasis in prostate cancer. *Oncogene* **19**:1288-1296.

Ko, J., Ou, S. and Patterson, P.H. (2001) New anti-huntingtin monoclonal antibodies: Implications for huntingtin confirmation and its binding proteins. *Brain Res. Bull*. **56**:319-329.

Lo, E., Dormand, E., Greenwood, A. and Anderson, D.J. (2002) Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian achaete-scute and atonal homologs in cultured neural progenitor cells. *Development* **129**:1553-1557.

Schindelholz, B., Knirr, M., Warrior, R. and Zinn, K. (2001) Regulation of CNS and motor axon guidance in *Drosophila* by the receptor tyrosine phosphatase DPTP52F. *Development.* **128**:4371-4382.

Van Der Most, R.G., Murali-Krishna, K., Ahmed, R. and Strauss, J.H. (2000) Chimeric yellow fever/dengue virus as a candidate dengue vaccine: Quantitation of the dengue virus-specific CD8 T-cell response. *J. Virol.* **74**:8094-8101.

Walikonis, R.S., Jensen, O.N., Mann, M., Provance, D.W., Mercer, J.A. and Kennedy, M.B. (2000) Identification of proteins in the postsynaptic density fraction by mass spectrometry. *J. Neurosci.* **20**:4069-4080.

Walikonis, R.S., Oguni, A., Khorosheva, E.M., Jeng, C., Asuncion, F.J. and Kennedy, M.B. (2001) Densin-180 forms a ternary complex with the alpha-subunit of Ca^{2+}/calmodulin-dependent protein kinase II and alpha-actinin. *J. Neurosci*. **21**:423-433.

Nucleic Acid and Protein Sequence Analysis Computing Facility

Supervisor: Stephen L. Mayo
Staff: David Mathog

The Sequence Analysis Facility (SAF) provides software, computers, and support for the analysis of nucleic acid and protein sequences. Currently, the SAF hardware consists of a Sun Netra running Solaris (mendel.bio.caltech.edu), a small Beowulf cluster of nine Linux/Alpha nodes, a 32-page per minute duplexing laser printer, and a color laser printer. The Biology Division's slidemaker, and the SGI and Windows NT workstations that comprise the "structure analysis facility" are also located in our facility. The SAF has over 250 registered users distributed among 50 research groups.

Most common programs for sequence analysis are available on Mendel. These include the GCG and EMBOSS Packages, PRIMER3, Phred, Phrap, Cross_Match, Phylip, and HMMER. Many of these may be accessed through the W2H or Pise web interfaces. Users may supply their own sequences or use those in the locally-maintained databases (Genbank, PIR, SwissProt, and others). Other programs, custom written programs, or special databases are available on request. Programs on the SGI workstations include InsightII, O, MidasPlus, Setor, Rasmol, VMD, Molscript, XtalView, CCP4, Delphi and GRASP. Structures may also be manipulated with Swiss PDB Viewer on the Windows NT workstations. All workstations support hardware stereo. The documentation for these programs is available on the SAF web server (http://saf.bio.caltech.edu/) and has been indexed for keyword searching. The lecture notes and homework from the introductory course "Fundamentals of Sequence Analysis" are also available on the SAF web server. The SAF Beowulf cluster runs BLAST searches (in parallel) more rapidly than does the NCBI server. BLAST jobs may be submitted through the SAF web server.

Nucleic acid sequences from the DNA sequencing facility are distributed through the SAF web and FTP servers. This year the web interface has been enhanced. In addition to the restriction digest, sequence difference, and printing options that were already available, it is now also possible to click on a result sequence and generate sequencing primers from it directly. Similarly, a few mouse clicks will submit a sequence to the SAF Beowulf for a BLAST search against a sequence or CDD database.

Protein Expression Center
Supervisor: Pamela Bjorkman
Director: Peter M. Snow
Staff: Cynthia Jones, Inderjit Nangiana

The Protein Expression Center was established in 1995 with the intent of providing services related to the expression of recombinant proteins to the Caltech community, as well as to investigators outside of the Institute. Services provided range from generation of recombinant DNA constructs to expression of recombinant proteins to purification of the expressed molecules.

The tissue culture portion of the facility, which is central to the operation, is equipped with an inverted phase contrast microscope designed for examination of cells, two laminar flow hoods suitable for sterile manipulations, two incubators capable of supporting the growth of mammalian cell lines, and two refrigerated incubators housing eight spinner plates which are used for the growth of insect cells. The center is currently capable of small to medium scale (100 ml to 10 liter scale) production of recombinant proteins in the baculovirus system, which is the most widely used eukaryotic system for the production of recombinant proteins. We have also explored the use of a second eukaryotic expression system (the methylotropic yeast *Pichia pastorius*), which has the potential to generate large amounts of recombinant proteins. The Center is currently able to generate a minimum of 10 liters/week of infected cells in the baculovirus system using a spinner flask and shaker flask configurations. A 15-liter bioreactor, which is suitable for larger scale production of proteins expressed in baculovirus as well as other eukaryotic systems (such as mammalian cells), is also available. A second 15-liter fermenter gives us the capability to grow both bacteria and yeast in volumes up to 10 liters. In addition, smaller quantities of both bacteria and yeast (50 ml-2 liters) may be grown in our shaking incubator. We are also capable of growing mammalian cell lines on a more limited basis (25 ml-500 ml).

The Expression Center is equipped to perform most common techniques in molecular biology (including PCR, construction and analysis of recombinant DNA vectors, and molecular cloning of DNA molecules). In addition, we are capable of protein concentration, protein purification (FPLC), and analysis using standard biochemical techniques (for example, most types of electrophoresis). In the current fiscal year, we also purchased a second refrigerated centrifuge, situated in BI286. This has allowed us to isolate expression performed in bacterial and yeast systems from that done in eukaryotic systems (performed solely in BI268), thereby minimizing the possibility of contamination.

To date, during the 2001/2002 fiscal year, the Center has expressed more than 50 different proteins in varying quantities (requiring the generation of approximately 230 liters of baculovirus-infected insect cells, 2 liters of bacterial cells and 2 liters of recombinant yeast) for a number of investigators both at Caltech and outside of the Institute. During this period, we have made 49 new recombinant viruses, with the recombinant DNA used for the generation of 25 of these viruses being constructed at the Center. In addition, 30 of the expressed proteins were concentrated and at least partially purified by Expression Center personnel for the investigators. We have also expressed five recombinant proteins in bacteria, and purified the expressed proteins.

Examples of the types of proteins that have been expressed and their intended uses include secreted molecules for crystallography (Pamela Bjorkman and James Strauss), or functional studies (David Anderson and Kai Zinn), cytoplasmic proteins for crystallography (David Chan) or functional studies (Judy Campbell, Richard Roberts, Mary Kennedy, Alexander Varshavsky, Linda Hsieh-Wilson) and cell surface channels for biophysical analyses (J. Nadeau, JPL). In addition, a number of proteins have been expressed for use as immunogens for the generation of antibodies (Marianne Bronner-Fraser, Alexander Varshavsky, Kai Zinn).

The Center has also provided services to the following: Peter Christmas at Massachusetts General Hospital; Chand Desai at Vanderbilt University; Randall Davis at the University of Alabama at Birmingham; Caroline A. Enns at Oregon Health Sciences University; Joyce Fingeroth at Harvard Medical School; Richard J. Kuhn at Purdue University; Richard LeBaron at the University of Texas; Chih-Pin Liu at the City of Hope Hospital; Anne Mason at the University of Vermont College of Medicine; Malini Raghavan at The University of Michigan Medical School; Cynthia Roy at Harvard Medical School; Jay Subramanian at the University of Florida; Matt Welch at the University of California at Berkeley; and Olga Zak at the Albert Einstein College of Medicine.

Publications

Dua, R., Levy, D.L., Li, C., Snow, P. and Campbell, J.L. (2002) *In vivo* reconstitution of *Saccharomyces cerevisiae* DNA polymerase in insect cells: Purification and characterization. Manuscript in preparation.

Lo, L., Dormand, E., Greenwood, A. and Anderson, D.J. (2002) Comparison of the generic neuronal differentiation and neuron subtype specification functions of mammalian *achaete-scute* and *atonal* homologs in cultured neural progenitor cells. *Development* **129**:1553-1567.

Mancino, L., Rizvi. S.M., Lapinski, P.E and Raghavan, M. (2002) Calreticulin recognizes misfolded HLA-A2. *Proc. Natl. Acad. Sci. USA.* In press.

Schindelholz, B., Knirr, M., Warrior, R. and Zinn, K. (2001) Regulation of CNS and motor axon guidance in *Drosophila* by the receptor tyrosine phosphatase DPTP52F. *Development* **128**:4371-4382.

Subramanian, J., Li, Z.J. and Gray D.J. (2002) Constitutive expression of PR-5 proteins after *in vitro* selection and their role in anthracnose resistance in grapevine. *Plant Physiology*. Manuscript in preparation.

Sun, Q., Schindelholz, B., Knirr, M., Schmid, A. and Zinn, K. (2001) Complex genetic interactions among four receptor tyrosine phosphatases regulate axon guidance in *Drosophila*. *Mol. Cell. Neurosci*. **17**:274-291.

West, A.P., Jr., Giannetti, A.M., Herr, A.B., Bennett, M.J, Nangiana, J.S., Pierce, J.R., Snow, P.M. and Bjorkman, P.J. (2001) Mutational analysis of the transferrin receptor reveals overlapping HFE and transferrin binding sites. *J. Mol. Biol*. **313**:385-397.

The Protein Microanalytical Laboratory
Director: Gary M. Hathaway, Ph.D.
Associate Biologist: Felicia Rusnak, B.S.
Senior Research Assistant and MPS: Dr. Jie Zhou, Ph.D.
Faculty Advisor: William G. Dunphy, Ph.D.
http://www.its.caltech.edu/~ppmal, or
http://www.caltech.edu/subpages/analyt.html or
http://www.its.caltech.edu/~bi/bicatalog.html#ppmal

Summary: The Protein Micro Analytical Laboratory (PPMAL) performs structure analysis on proteins, peptides and other biopolymers. The laboratory also develops new methodologies and techniques for application in the area of biopolymer analysis. Partially funded by the Beckman Institute, charges are set to recover residual costs. The laboratory is located in room 204 with offices in rooms 232 and 215 of the Beckman Institute, 626-395-6388/2769, Fax: 626-449-3414.

Activity Centers

Biopolymer Mass Spectrometry

Tandem mass spectrometry peptide sequencing
Capillary liquid chromatography coupled mass and tandem mass spectrometry
Matrix-assisted, laser desorption, time-of-flight mass spectrometry
Post source decay and prompt fragmentation analysis
In-gel digestion for mass mapping and sequencing by mass spectrometry
Fragmentation analysis by database search for protein identification
Post-translational and chemical modification analysis

Edman Sequencing

N-terminal sequence analysis of proteins and peptides by Edman degradation with capillary chromatography

High Performance Liquid Chromatography

Submillimeter and capillary HPLC for analysis and purification

Specialty Analyses

De novo sequence analysis of tandem mass spectrometry-derived data
Chemical modification of proteins and peptides for mass spectrometry. Including acetylation, O-methylisourea conversion of lysine residues, chemical modification of phospho- and glyco-peptides for mass and chemical sequence analysis
Consultation on sample preparation and error analysis including dissemination of literature materials
Database search by sequence, fragment mass, and composition

SPECIFICS

Mass Spectrometry of Biopolymers: The facility operates two mass spectrometers: a triple quadrupole, tandem mass spectrometer, and the time-of-flight mass spectrometer. The liquid chromatography (LC/MS) interface designed by our lab is coupled to a technique (also invented by the PPMAL) for gradient elution using static mini-gradients. We construct the reverse phase columns for use within an electrospray needle housing of our design (2). With this methodology we have obtained good MS/MS fragmentation spectra with as little as 10 femtomoles of peptides (3).

Protein/Peptide Chemical Sequence Analysis by Edman Degradation

We use a dual reaction column, capillary microsequencer with subpicomole sensitivity. As demonstrated (3), the new instrument extends the use of the technology for combined chemical and MS sequencing projects. We have also modified the chemistry to improve performance (4).

Chemical Modification of Proteins and Peptides

We chemically modify peptides particularly those with post-translational modifications to facilitate structural analysis. As part of our mission, the facility publishes original research efforts and makes the new applications available to the campus.

Database Search and Analysis

The laboratory maintains access to database search via the web with programs such as MASCOT, BLAST, and MSA using information obtained from mass and chemical sequencing. Searches often use mass information achieved by collisional fragmentation and predictive software (GPMAW and SHERPA). They are available on our lab computers as independent software, and through the Internet.

Collaborations and Accomplishments

During the course of the year the lab interacted with 28 laboratories, their professional staff and associates. We assisted local biotechnology companies associated closely with Caltech such as Xencor, Clinical Microsencors, and Insert Therapeutics. Additionally, faculty are assisted through our efforts on behalf of the Biopolymer Synthesis and Protein Expression Facilities. Information regarding sample preparation, cost recovery schedules, and other technical aspects of our operation can be accessed through our homepage (http://www.its.caltech.edu/~ppmal). The

web site also provides the reader with direct access for comments and questions. We can be reached by email at: ppmal@its.caltech.edu or by calling 626-395-6388.

Future Expectations and Directions

Our laboratory continuously strives to develop new procedures for high throughput analysis while maintaining high sensitivity. Results are reflected in short turnaround times with recovery costs competitive with other premier institutions. To date, the facility has completed more than 22,000 analyses.

Publications

(1) Rusnak, F., Zhou, J. and Hathaway, G. (2002) Identification of phosphorylated and glycosylated sites in peptides by chemically targeted proteolysis. Accepted - *J. Biomol. Tech.*

(2) Zhou, J., Rusnak, F., Colonius, T. and Hathaway, G.M. (2000) Quasi-linear gradients for capillary liquid chromatography with mass and tandem mass spectrometry. *Rapid Commun. Mass Spectrom.***14**:432-438.

(3) Hathaway, G.M. (2000) Direct gradient-elution of peptides for capillary LC/MS/MS application to the ABRF-00SEQ protein sequencing test peptide. In: "Vydac Advances," summer issue.

(4) Rusnak, F. and Hathaway, G. (2001) Application of a coupled reducing system to Edman sequencing. *J. Biomol. Tech.* **12**:40-43.

Graduates

Bachelor of Science – 2002

Brian Robert Blood
Fernando Alonso Campos
Elisa Ka Yee Chan*
Sunny Tak Cheung Chan
Wendy M. Ching*
Nalini Anne Colasco*
Karen Marie Daugherty*
Cheryl Ann Forest
Giao Bich Hang
Garrett Collins Heffner*
Elizabeth Jennifer Hong*
Barbara Karmen Kraatz
Thea Lu
Sarah Jane Mahoney
Florian Tobias Merkle*
Albert Tuong-Quang Nguyen*
Madeleine Emily Price*
Richard Aaron Robison*
Robb Brooks Rutledge*
Derek Michael Shannon
Anongpat Suttangkakul*
Jennifer Patricia Tung*
Emily Wang
Jessica Lynn Yohe

*Students whose names are followed by an asterisk have graduated with Honor in accordance with a vote of the faculty.

Master of Science – 2002

Loretta Yvonne Hidalgo

Sudipta Bardhan Quallen

Doctor of Philosophy - 2002

Girish Nanda Aakalu — *Biology*
B.A., Johns Hopkins University, 1996
M.S., California Institute of Technology, 1998
Thesis: Building the Molecular Machinery of Memory: Local Protein Synthesis in Hippocampal Neurons.

Benjamin Jacob Arthur — *Computation and Neural Systems*
B.S., Trinity University, 1992
Thesis: Neural Computations Leading to Space-specific Auditory Responses in the Barn Owl.

Alejandro Bäcker — *Biology and Computational and Neural Systems*
S.B., Massachusetts Institute of Technology, 1995
M.S., California Institute of Technology, 1998
Thesis: Priming, Gain Control and Coding Issues in the Locust Olfactory System.

Daniel N. Bolon — *Biochemistry and Molecular Biophysics*
B.S., Duke University, 1997
Thesis: Computational Enzyme Design.

Tianxin (Cynthia) Chen — *Biochemistry and Molecular Biophysics*
B.S., Tsinghua University, 1996
Thesis: Regulatory Mechanisms of the Heat Shock Response.

Wonchae Choe — *Biology*
B.S., Kyung Hee University, 1992
M.S., 1994
M.S., California Institute of Technology, 2000
Thesis: Biochemical and Biological *in vivo* Functions of Dna2p in *Saccharomyces cerevisiae.*

Fangyong Du — *Biology*
B.S., Peking University, 1991
M.S., Peking University, 1994
Thesis: Allosteric Activation of the Ubiquitin Ligase UBR1 by Short Peptides: Molecular Mechanisms and Physiological Functions.

David Julian Dubowitz — *Computation and Neural Systems*
B.A., University of Cambridge, 1985
M.A., 1989
B.M., University of Oxford, 1988
Thesis: Functional Magnetic Resonance Imaging in Rhesus Macaque Monkeys.

Sebastian de la Soudière Gerety — *Biology*
B.S., Tufts University, 1992
Thesis: Eph Signaling in Vascular Development.

Zsuzsa Andrea Hamburger — *Biology*
B.S., Purdue University, 1996
Thesis: Crystallographic Studies of Invasin, a Bacterial Adhesion Molecule from *Yersinia pseudotuberculosis*.

Gabriel Kreiman — *Biology*
B.Sc., University of Buenos Aires, 1996
Thesis: On the Neuronal Activity in the Human Brain during Visual Recognition, Imagery and Binocular Rivalry.

Anthony Leonardo — *Computation and Neural Systems*

B.S., Carnegie Mellon University, 1994
Thesis: Neural Dynamics Underlying Complex Behavior in a Songbird.

Tanya Munnecke Moreno — *Biology*

B.S., University of California, San Diego, 1992
Thesis: *Noelin* in Neural Development.

David Rosenbluth — *Computation and Neural Systems*

A.B., Columbia University, 1988
M.S., New York University, 1991
Thesis: Eye Position Modulation of Visual Cortex and the Sensory Set Hypothesis.

David J. Shuey — *Biochemistry and Molecular Biophysics*

B.S., Pennsylvania State University, 1982
Thesis: A Detailed Analysis of the DNA Binding Properties and the Affinity Purification of the *Drosophila* Heat-Shock Transcription Factor.

Pavel Strop — *Biochemistry and Molecular Biophysics*

B.S., University of Arizona, 1997
Thesis: Characterization of the Mechanosensitive Channel of Large Conductance.

Stephanie Yeager Vernooy — *Biology*

B.A., Pomona College, 1994
M.S., California Institute of Technology, 2000
Thesis: Identification of Apoptotic Regulators in *Drosophila* and their Nonapoptotic Roles in Spermatogenesis: Implications for the Existence of a "Caspase Cassette" which Regulates Diverse Biological Processes.

Mariela Zirlinger — *Biology*

B.S., University of Buenos Aires, 1996
Thesis: Application of Microarray, Laser Capture and Transgenic Technologies to the Study of Neural Diversity.

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Financial Support

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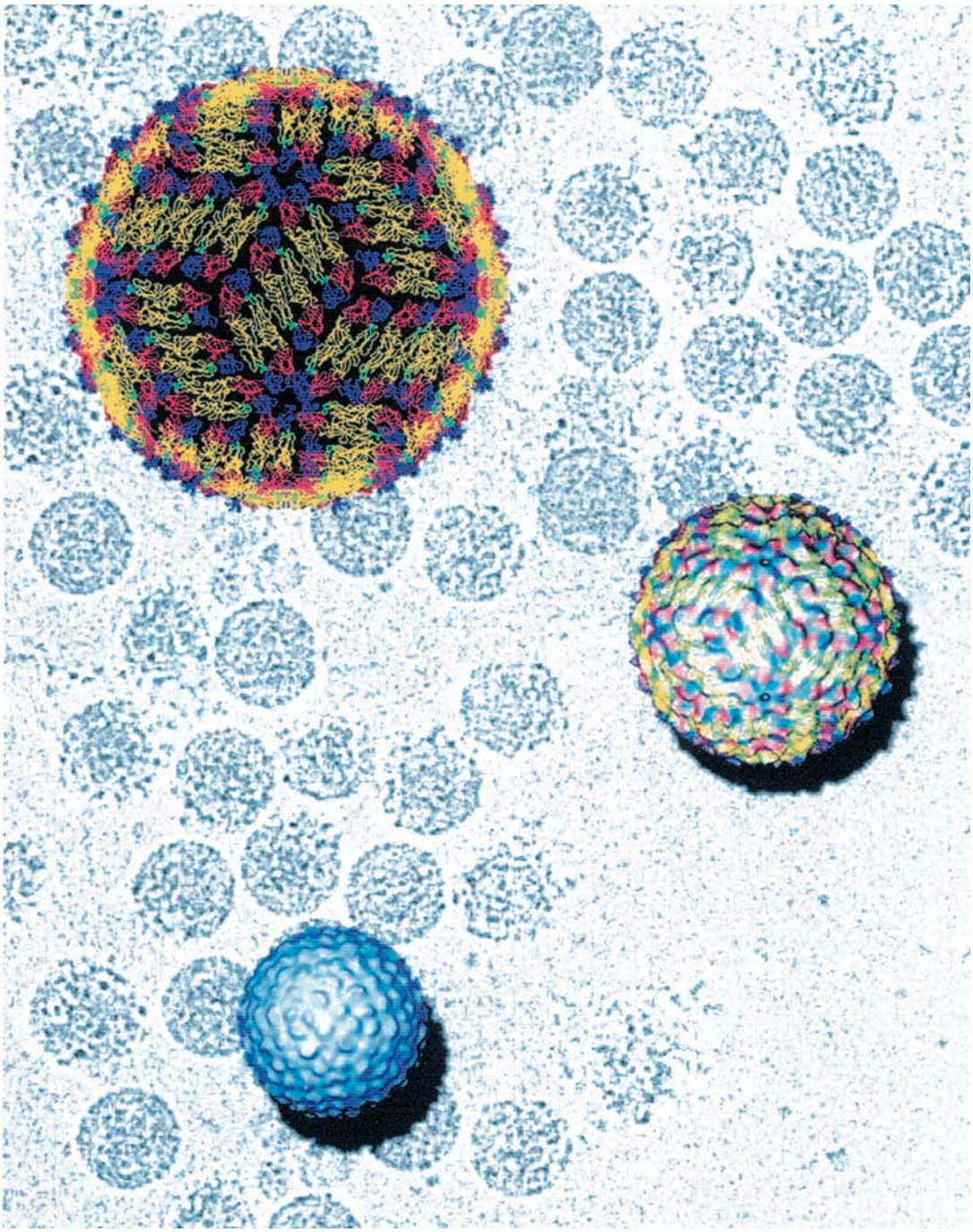

Strauss Group in collaboration with scientists at Purdue University

Structure of Dengue Virus

This montage illustrates the first structure determined for a flavivirus (Kuhn *et al*. (2002) *Cell* **108**:717-725). In the largest particle (upper left), the atomic structure of the envelope glycoproteins of a flavivirus has been fitted into the cryoEM density of a dengue particle. The E protein is composed of three domains, rendered in three different colors. The 180 E proteins are present as 90 dimers, and do not all occupy quasi-equivalent positions. In the smallest sized particle (lower left), a surface shaded representation of a dengue particle is shown, as determined by cryoEM. The intermediate sized particle (center right) is a composite of these two representations that shows how they are related. In the background are seen dengue virions imaged by cryoEM. (See Abstract 268 of last year's Annual Report and the Strauss Group abstracts starting on page 183)