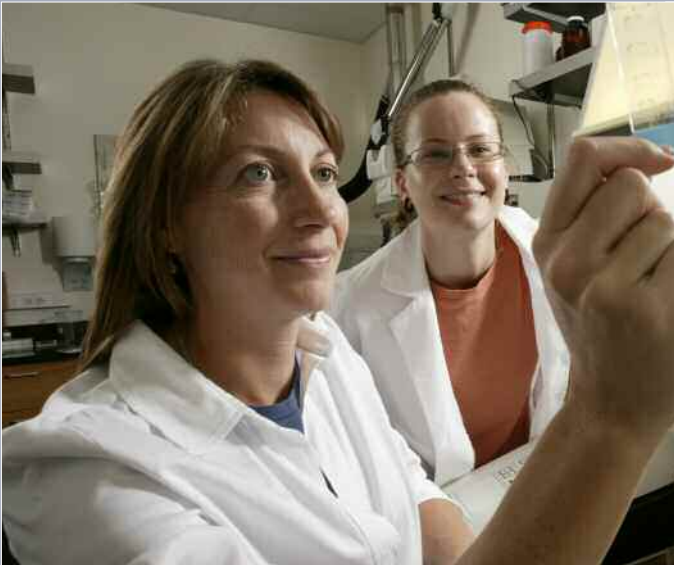


## Cell Biology



Three-dimensional reconstruction of the upper half of a 3-somite-pair mouse embryo stained by whole-mount immunofluorescence with tropomodulin 1 (green), F-actin (red), and Hoechst dye (blue). Work done by Roberta Nowak, research assistant, in the laboratory of Velia M. Fowler, Ph.D., professor.



*Lisa Stowers, Ph.D., Assistant Professor, and  
Kelly Flanagan, Graduate Student*

**DEPARTMENT OF CELL  
BIOLOGY****STAFF**

**Sandra L. Schmid, Ph.D.\***  
Professor and Chairman

**Francisco Asturias, Ph.D.\*\***  
Associate Professor

**William E. Balch, Ph.D.\***  
Professor

**Kristin Baldwin, Ph.D.\*\*\***  
Assistant Professor

**Bridget Carragher, Ph.D.\*\***  
Associate Professor

**Benjamin Cravatt, Ph.D.\*\*\*\***  
Professor  
Director, Helen L. Dorris  
Child and Adolescent Neuro-  
Psychiatric Disorder Institute

**Gaudenz Danuser\*\***  
Associate Professor

**Philip E. Dawson, Ph.D.\*\*\*\*\***  
Associate Professor

**Velia Fowler, Ph.D.\*\***  
Professor

**Martin Friedlander, M.D.,  
Ph.D.**  
Professor

**Larry R. Gerace, Ph.D.\***  
Professor

**Shelley Halpain, Ph.D.\*\*\***  
Associate Professor

**Natasha Kralli, Ph.D.**  
Associate Professor

**Peter Kuhn, Ph.D.\*\***  
Associate Professor

**David Loskutoff, Ph.D.**  
Professor Emeritus

**Mari Manchester, Ph.D.\*\***  
Associate Professor

**Stephen P. Mayfield,  
Ph.D.\*\*\*\*\***  
Professor

**Mark Mayford, Ph.D.\*\*\***  
Associate Professor

**Lindsey Miles, Ph.D.**  
Associate Professor

**Ronald A. Milligan, Ph.D.\*\***  
Professor

**Ulrich Müller, Ph.D.\*\*\***  
Professor

**Ardem Patapoutian, Ph.D.†**  
Associate Professor

**Clinton Potter, B.S.\*\***  
Associate Professor

**James Quigley, Ph.D.**  
Professor

**Enrique Saez, Ph.D.**  
Assistant Professor

**Lisa Stowers, Ph.D.††**  
Assistant Professor

**Peter N. T. Unwin, Ph.D.\*\***  
Professor

**Clare Waterman-Storer,  
Ph.D.†††**  
National Heart, Lung, and  
Blood Institute  
Bethesda, Maryland

**Elizabeth Winzeler, Ph.D.†**  
Associate Professor

**John R. Yates III, Ph.D.**  
Professor

**Mark J. Yeager, M.D., Ph.D.**  
Professor

**ADJUNCT APPOINTMENTS**

**Alan Bell, B.S.C.S.**  
Xerox Palo Alto Research  
Center  
Palo Alto, California

**Kelly Bethel, M.D.**  
Scripps Clinic Medical Group  
La Jolla, California

**Alan Bird, M.D.**  
Institute of Ophthalmology  
London, England

**Richard Bruce, Ph.D.**  
Xerox Palo Alto Research  
Center  
Palo Alto, California

**Douglas Curry, B.S. (E.E.C.S.)**  
Xerox Palo Alto Research  
Center  
Palo Alto, California

**Bertil Daneholt, M.D.**  
Karolinska Institutet  
Stockholm, Sweden

**Scott Elrod, Ph.D.**  
Xerox Palo Alto Research  
Center  
Palo Alto, California

**Mark Ginsberg, M.D.**  
University of California  
San Diego, California

**David Goldberg, Ph.D.**  
Xerox Palo Alto Research  
Center  
Palo Alto, California

**Xiaohua Gong, Ph.D.**  
University of California  
Berkeley, California

**Klaus Hahn, Ph.D.**  
University of North Carolina  
Chapel Hill, North Carolina

**Eric Peeters, Ph.D.**  
Xerox Palo Alto Research  
Center  
Palo Alto, California

**Heidi Stuhlmann, Ph.D.**  
Weill Medical College of  
Cornell University  
New York, New York

**STAFF SCIENTISTS**

**Michael Bracey, Ph.D.**

**Anchi Cheng, Ph.D.**

**Elena Deryugina, Ph.D.**

**Robert Fischer, Ph.D.**

**Lesley Page-Chappel, Ph.D.**

**Matthew Ritter, Ph.D.**

**Elizabeth Wilson, Ph.D.**

**SENIOR RESEARCH  
ASSOCIATES**

**Brian Adair, Ph.D.**

**Barbara Calabrese, Ph.D.**

**Mark Daniels, Ph.D.**

**Jeremiah Joseph, Ph.D.**

**Martin Schwander, Ph.D.**

**Defne Yarar, Ph.D.**

**Andries Zijlstra, Ph.D.†††**  
Vanderbilt University  
Nashville, Tennessee

**RESEARCH ASSOCIATES**

**Jessica Alexander, Ph.D.†††**  
Merck Pharmaceuticals  
Whitehouse Station, New  
Jersey

**Geza Ambrus-Aikelin, Ph.D.**

**Veronica Ardi, Ph.D.**

**Andrea Bacconi, Ph.D.**

**Hedieh Badie, Ph.D.†††**  
Burnham Institute for  
Medical Research  
San Diego, California

**Hongdong Bai, Ph.D.**

<b>Kent Baker, Ph.D.</b>	<b>Anouk Dirksen, Ph.D.</b>	<b>Nobutaka Kato, Ph.D.</b>	<b>Isabel Martinez-Garay, Ph.D.</b>
<b>Michael Bandell, Ph.D.</b>	<b>Meng-Qui Dong, Ph.D.</b>	<b>Jin Young Kim, Ph.D.</b>	<b>Julia Navarro, Ph.D.</b>
<b>Claudia Barros, Ph.D.</b>	<b>Jonas Dorn, Ph.D.</b>	<b>Katsuhiko Kita, Ph.D.</b>	<b>Michael Matho, Ph.D.</b>
<b>Juan Bautista, Ph.D.</b>	<b>Michael Dorrell, Ph.D.</b>	<b>Kevin Koehntop, Ph.D.</b>	<b>Naoki Matsuo, Ph.D.</b>
<b>Maria Beligni, Ph.D.</b> <sup>†††</sup> Rincon Pharmaceuticals La Jolla, California	<b>Kelly A. Dryden, Ph.D.</b>	<b>Jenny Kohler, Ph.D.</b> <sup>†††</sup>	<b>Daniel McClatchy, Ph.D.</b>
	<b>Jerome Dupuy, Ph.D.</b>	<b>Atanas Koulov, Ph.D.</b>	<b>Caroline McKeown, Ph.D.</b>
<b>Richard Belvindrah, Ph.D.</b>	<b>Anna Durrans, Ph.D.</b> <sup>†††</sup> Cornell University Ithaca, New York	<b>Shailendra Kumar, Ph.D.</b>	<b>Stephan Meister, Ph.D.</b>
<b>Pablo Chamero Benito, Ph.D.</b>		<b>Jean-Cheng Kuo, Ph.D.</b> <sup>†††</sup> National Heart, Lung, and Blood Institute Bethesda, Maryland	<b>Marcel Mettlen, Ph.D.</b>
<b>Edward Brignole III, Ph.D.</b>	<b>Santos Franco, Ph.D.</b>		<b>Jennifer Mitchell, Ph.D.</b>
<b>Florence Brunel, Ph.D.</b>	<b>Margaret Gardel, Ph.D.</b> <sup>†††</sup> University of Chicago Chicago, Illinois	<b>Paul LaPointe, Ph.D.</b>	<b>Nico Mitro, Ph.D.</b>
<b>Gang Cai, Ph.D.</b>		<b>Nicole Lazarus, Ph.D.</b>	<b>Machiko Muto, Ph.D.</b>
<b>Gregory Cantin, Ph.D.</b> <sup>†††</sup> Dow Chemical Company San Diego, California	<b>Mohsen Sabouri Ghomi, Ph.D.</b>	<b>Philip Lee, Ph.D.</b>	<b>Ryuichiro Nakai, Ph.D.</b>
	<b>Jorg Grandl, Ph.D.</b>	<b>Raymond Lewis, Ph.D.</b>	<b>Aleksey Nakorchevskiy, Ph.D.</b>
<b>Eric Carlson, Ph.D.</b>	<b>Nicolas Grillet, Ph.D.</b>	<b>Lujian Liao, Ph.D.</b> <sup>†††</sup> Emory University Medical School Atlanta, Georgia	<b>Andromeda Nauli, Ph.D.</b>
<b>Aurelia Cassany, Ph.D.</b>	<b>Bin Guo, Ph.D.</b> <sup>†††</sup> University of California Berkeley, California		<b>Claire Tiraby Nguyen, Ph.D.</b>
<b>Yuriy Chaban, Ph.D.</b>		<b>Jennifer Lin, Ph.D.</b> <sup>†††</sup> Salk Institute for Biological Studies La Jolla, California	<b>Sherry Niessen, Ph.D.</b>
<b>Emily Chen, Ph.D.</b>	<b>Stephanie Gupton, Ph.D.</b> <sup>†††</sup> Massachusetts Institute of Technology Cambridge, Massachusetts	<b>Dinah Loerke, Ph.D.</b>	<b>Yukako Nishimura, Ph.D.</b> <sup>†††</sup> National Heart, Lung, and Blood Institute Bethesda, Maryland
<b>Yei Hua Chen, Ph.D.</b>	<b>Xuemei Han, Ph.D.</b>	<b>Darren Logan, Ph.D.</b>	<b>Bryan O'Neill, Ph.D.</b>
<b>Smita Chitnis, Ph.D.</b> <sup>†††</sup> Cornell University Ithaca, New York	<b>Johannes Hewel, Ph.D.</b> <sup>††††</sup>	<b>Bingwen Lu, Ph.D.</b>	<b>Silvia Ortega-Gutierrez, Ph.D.</b> <sup>†††</sup> Universidad Complutense Madrid, Spain
<b>Esther Choi, Ph.D.</b>	<b>Michael Hock, Ph.D.</b>	<b>Matthias Machacek, Ph.D.</b> <sup>†††</sup> Novartis AG Basel, Switzerland	<b>Sandra Pankow, Ph.D.</b>
<b>Parag Chowdhury, Ph.D.</b> <sup>††††</sup>	<b>Ke Hu, Ph.D.</b> <sup>†††</sup> Indiana University Bloomington, Indiana	<b>Kalotina Machini, Ph.D.</b>	<b>Fabio Papes, Ph.D.</b>
<b>Jill Chrencik, Ph.D.</b>		<b>Mark Madsen, Ph.D.</b> <sup>†††</sup> Biogen Idec San Diego, California	<b>Ana Maria Pasapera, Ph.D.</b> <sup>†††</sup> National Heart, Lung, and Blood Institute Bethesda, Maryland
<b>Michael Churchill, Ph.D.</b>	<b>Michael Huber, Ph.D.</b>	<b>Valentina Marchetti, Ph.D.</b>	<b>Joanna Pawlak, Ph.D.</b>
<b>Francesco Conti, Ph.D.</b>	<b>Darren Hutt, Ph.D.</b>	<b>Brent Martin, Ph.D.</b>	<b>Per Pettersson, Ph.D.</b>
<b>Judith Coppinger, Ph.D.</b>	<b>Eric Hwang, Ph.D.</b>		
<b>Kaustuv Datta, Ph.D.</b>	<b>Khuloud Jaqaman, Ph.D.</b>		
<b>Leif Dehmelt, Ph.D.</b>	<b>Lin Ji, Ph.D.</b>		
<b>Ajay Dhaka, Ph.D.</b>			

**Sergey Plotnikov, Ph.D.**<sup>†††</sup>

National Heart, Lung, and  
Blood Institute  
Bethesda, Maryland

**Barbie Pornillos, Ph.D.****Anita Pottekat, Ph.D.****Judith Prieto, Ph.D.****Thomas Pucadyil, Ph.D.****Rajesh Ramachandran, Ph.D.****Vandana Ramachandran,  
Ph.D.****Abbas Razvi, Ph.D.****Leon Reijmers, Ph.D.****Anna Reynolds, Ph.D.****Cristian Ruse, Ph.D.****Kumar Saikatendu, Ph.D.****Cleo Salisbury, Ph.D.****Manuela Schmidt, Ph.D.****Ian Schneider, Ph.D.****Christina Schroeder, Ph.D.**<sup>††††</sup>**Pratik Singh, Ph.D.**<sup>†††</sup>

University of California  
Irvine, California

**Scott Stagg, Ph.D.****Mark Surka, Ph.D.**<sup>†††</sup>

Bristol-Myers Squibb Co.  
New York, New York

**Patricia Szainer, Ph.D.****Ingo Thievensen, Ph.D.**<sup>†††</sup>

National Heart, Lung, and  
Blood Institute  
Bethesda, Maryland

**Valerie Uzzell, Ph.D.****John Venable, Ph.D.**<sup>†††</sup>

Novartis  
Boston, Massachusetts

**Josep Villena, Ph.D.**<sup>†††</sup>

Fundació Institut de Recerca  
de l'Hospital Universitari  
Vall d'Hebron  
Barcelona, Spain

**Neil Voss, Ph.D.****Kari Bradtke Weber, Ph.D.**<sup>†††</sup>

University of Wisconsin-  
Madison  
Madison, Wisconsin

**Eranthie Weerapana, Ph.D.****BinQing Wei, Ph.D.****Scott Westenberger, Ph.D.****Ann Wheeler, Ph.D.**<sup>†††</sup>

Imperial College London  
London, England

**James Wohlschlegel, Ph.D.**<sup>†††</sup>

University of California  
Los Angeles, California

**Catherine Wong, Ph.D.****Charmian Cher Wong, Ph.D.****Aaron Wright, Ph.D.****Beili Wu, Ph.D.****Ge Yang, Ph.D.****Bailong Xiao, Ph.D.****Wei Xiong, Ph.D.****Fan Zhang, Ph.D.****Qiang Zhao, Ph.D.****SCIENTIFIC ASSOCIATES****Hilda Edith Aguilar de Diaz,  
M.D.****Alexei Brooun, Ph.D.**<sup>††††</sup>**Claire Delahunty, Ph.D.****Mohammed El-Kalay, Ph.D.****Tinglu Guan, Ph.D.****Anand Kolatkar, Ph.D.****Tatyana Kupriyanova, Ph.D.****Lujian Liao, Ph.D.**

\* Joint appointment in the  
Department of Molecular Biology

\*\* Joint appointment in the Center  
for Integrative Molecular  
Biosciences

\*\*\* Joint appointment in the  
Institute for Childhood and  
Neglected Diseases

\*\*\*\* Joint appointments in the  
Department of Chemistry, The  
Skaggs Institute for Chemical  
Biology, and the Helen L. Dorris  
Child and Adolescent Neuro-  
Psychiatric Disorder Institute

\*\*\*\*\* Joint appointment in The Skaggs  
Institute for Chemical Biology

† Joint appointments in the  
Institute for Childhood and  
Neglected Diseases and the  
Genomics Institute of the  
Novartis Research Foundation

†† Joint appointment in the Helen  
L. Dorris Child and Adolescent  
Neuro-Psychiatric Disorder  
Institute

††† Appointment completed; new  
location shown

†††† Appointment completed



*Sandra Schmid, Ph.D.*

## Chairman's Overview

Faculty members in the Department of Cell Biology continue to thrive, even in these difficult times of reduced funding from the National Institutes of Health. We remain highly competitive by focusing our efforts on the most important biological and medical questions and by taking innovative and interdisciplinary approaches to address these questions. Researchers in each laboratory have made substantial progress, as shown in the following pages. I recommend, in this computer age, that you supplement this report by visiting our faculty Web sites, which are linked to the list of cell biology faculty at <http://www.scripps.edu/cb/faculty>. These sites contain striking images that highlight research in the department and overviews that summarize the research directions of the faculty.

Recent findings from many investigators, which I briefly describe here, are directly relevant to human disease. Like the engineers of the 20th century who applied fundamental knowledge of physics, chemistry, and mathematics to technological advances, cell biologists in the 21st century are positioned to apply the ever-expanding fundamental knowledge of cell and molecular biology to deduce the root causes of human disease and reveal new therapeutic approaches for treatment.

Cystic fibrosis is a devastating disease caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR), which results in intracellular retention of the defective CFTR protein. Ironically, the mutant CFTR protein could do its job if it could only get to the cell surface where it normally functions. Bill Balch has discovered a protein involved in the intracellular retention of CFTR. Using the new technology of RNA interference, he found that reducing the cellular levels of the protein partially rescues the trafficking defect of mutant CFTR, releasing protein for surface expression. Similar strategies are being applied to understand other protein folding and trafficking defects that lead to Gaucher disease and amyloidoses, including Parkinson's and Alzheimer's diseases.

Ben Cravatt has developed tools to probe changes in enzymatic activities and the cell's metabolic state during cancer progression. Using this combination of "activity based" proteomics and "metabolomics," he can distinguish different types and stages of cancers and can reveal an Achilles heel that can be used to selectively target the cancers. For example, in collaboration with Jim Quigley, he identified protease activities specifically associated with aggressive metastatic cancer cells. Peter Kuhn and his colleagues at the Scripps-PARC Institute are developing the instrumentation and methods for detecting rare cancer cells in the circulation in humans. This technology could be a valuable new tool for cancer screening and for guiding individualized cancer therapies.

In an effort to develop HIV vaccines, Phil Dawson is using synthetic protein chemistry to design peptides that mimic HIV epitopes. He is using other methods to label proteins as imaging probes for positron emission tomography and magnetic resonance imaging for medical diagnostics. Larry Gerace has collaborated with John Yates to identify novel nuclear envelope transmembrane proteins. Mutations in these proteins are associated with numerous myelopathies, and Larry and his group are studying the function of these novel proteins by using muscle cells in culture in an effort to define potential causes of and treatments for these human diseases.

Natasha Kralli has identified a class of nuclear receptors that regulate mitochondrial function. Mitochondria are central to maintaining cellular energy levels, and their metabolic dysfunction is linked to type 2 diabetes, muscle degenerative diseases, and obesity. She is currently producing mice that lack these receptors to probe the functions of the receptors in muscle physiology and metabolism. Enrique Saez, a new faculty member in the

department, is studying another class of nuclear receptors that sense nutrient uptake and modulate metabolic pathways in response to diet. These receptors are novel therapeutic targets for a variety of metabolic diseases, including diabetes and obesity.

Mari Manchester and collaborators are developing versatile virus-based nanoparticles that can be used for sensitive whole-animal imaging studies, tumor targeting, and the effective presentation of antigens in vaccine development. Steve Mayfield is harnessing simple and inexpensively grown algae as “green cell factories” for the large-scale production of human recombinant antibodies and other proteins with therapeutic potential.

Mark Mayford has developed breakthrough technology whereby neurons activated by a behavioral stimuli can be permanently tagged and identified. Using this technology, he can map neuronal circuitry and determine whether the same neurons activated during learning are subsequently active during recall of the memory. This knowledge of brain function has implications in posttraumatic stress disorders and in a process called “extinction,” which is used in the treatment of phobias. Ardem Patapoutian and collaborators at the Genomics Institute of the Novartis Research Foundation have developed high-throughput screening methods for structure-function analysis of transient-receptor potential channels involved in temperature and pain sensation. Knowledge of mechanisms that govern the function of these channels is aiding in the development of small-molecule inhibitors as novel therapeutic agents for the treatment of pain.

On a more personal note, although members of the department are intensely focused on our research efforts, we also enjoy the collegial environment at Scripps Research. We are a young department and although the number of faculty may not have increased this year, the larger cell biology family at Scripps Research certainly has. The following faculty welcomed new babies into their families: Francisco Asturias, Ben Cravatt, Peter Kuhn, Natasha Kralli, Uli Müller, Tim Jegla, Shelley Halpain, and Lisa Stowers. We should not forget the importance of family and sharing the happiness of our colleagues.

## INVESTIGATORS' REPORTS

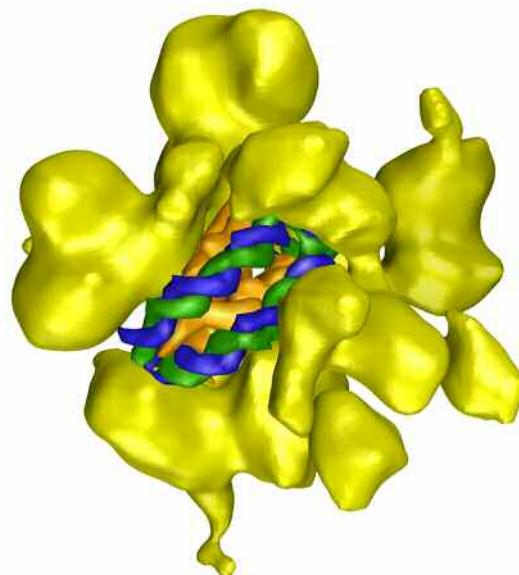
### Structural Characterization of Macromolecular Machines

F.J. Asturias, E. Brignole, J. Brown, G. Cai, Y. Chaban, J. Chittuluru, F. Xu, F. Zhang

**W**e use state-of-the-art electron microscopy and image analysis to determine the 3-dimensional structures of macromolecular complexes involved in a variety of cellular processes, including DNA transcription, DNA replication, chromatin modification and remodeling, and fatty acid synthesis. Macromolecular electron microscopy is ideal for these studies because it requires only a small amount of material and the conditions for preparing samples are physiologically relevant. Images of individual macromolecules are recorded and then computationally combined to obtain low to moderate (25–10 Å) resolution structures. These structures are often interpreted by docking atomic resolution structures of component subunits in the lower resolution map of an entire complex. Our ultimate goal is to use a combination of biochemical and structural information to reveal the mechanism by which a macromolecular complex carries out its function.

In our current studies on DNA transcription and its regulation, we are analyzing the basal machinery and assembly of the RNA polymerase II preinitiation complex. We are also studying complexes involved in the regulation of transcription during initiation and in earlier steps in which the structure of chromatin is altered to control access to DNA. We are particularly interested in the structure and function of an essential, abundant chromatin remodeling complex, RSC, purified from the yeast *Saccharomyces cerevisiae*. Electron cryomicroscopy analysis of RSC and of a RSC-nucleosome complex (Fig. 1) has led to an understanding of the RSC-nucleosome interaction that has important and general implications for the mechanism of chromatin remodeling.

Finally, we continue to investigate the role that conformational changes play in the function of mammalian fatty acid synthase (FAS), the enzyme responsible for the synthesis of long-chain fatty acids. In this true macromolecular assembly line, the different enzymes involved in fatty acid synthesis have fused into a single polypeptide chain that includes 6 catalytic and 1 acyl



**Fig. 1.** Independent electron cryomicroscopy reconstructions of RSC and an RSC-nucleosome complex were compared to determine the precise position of the nucleosome and to understand the effect of RSC binding on its structure. The nucleosome binds in a central cavity of the RSC complex. Upon binding, RSC-DNA interactions appear to destabilize the structure of the nucleosome core particle, causing the nucleosomal DNA to become partially disordered. Such rearrangement of the DNA could facilitate ATP-dependent sliding and nucleosome remodeling.

carrier protein domains. Using a novel approach in which FAS point mutants are imaged in the presence of substrates, we are able to “pause” FAS at a given catalytic step. Statistical analysis of images of single FAS particles is then used to sort tens of thousands of individual molecules into classes that show distinct FAS conformations, and sophisticated feature-tracking programs developed in the laboratory of G. Danuser, Department of Cell Biology, are used to obtain a quantitative description of FAS molecular motions. The final goal of this research is to provide a series of snapshots of FAS as it catalyzes different steps in fatty acid synthesis.

#### PUBLICATIONS

Takagi, Y., Calero, G., Komori, H., Brown, J.A., Ehrensberger, A.H., Hudmon, A., Asturias, F.J., Kornberg, R.D. Head module control of Mediator interactions. *Mol. Cell* 23:355, 2006.

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## Protein Homeostasis, Misfolding Disease, and Membrane Traffic

W.E. Balch, C. Chen, D. Hutt, W. Kellner, A. Koulov, P. LaPointe, J. Matteson, A. Murray, A. Nauli, L. Page, H. Plutner, A. Pottekat, A. Razvi, S. Stagg, P. Szajner, I. Yonemoto

**A** major challenge is to understand and treat the many protein-misfolding diseases that affect cellular protein-folding homeostasis (proteostasis) and human health, including cystic fibrosis, childhood emphysema, type 2 diabetes, and amyloidosis. These abnormalities are broadly classified as membrane-trafficking conformational diseases because a defect in protein folding or processing during transit through the eukaryotic secretory or exocytic pathway results in loss of normal host function. A key goal is to determine the underlying defect and define how that defect affects the ability of the protein to function normally within the context of the biological and chemical environments characteristic of the host cell and tissue type. Our broad objective is to define the basis for proteostatic diseases that involve the trafficking of misfolded proteins. We use chemical, structural, biological, and bioinformatics (systems level) approaches.

### PROTEIN TRAFFICKING

Eukaryotic cells are highly compartmentalized; each compartment of the exocytic and endocytic pathways provides a unique chemical and biological landscape in which protein folding and function can be modulated to maintain cellular proteostasis. Movement between these compartments involves the activity of both anterograde and retrograde transport tubules and vesicles. During export from the first compartment of the exocytic pathway, the endoplasmic reticulum, assembly of vesicle budding sites involves the coatamer complex II (COPII) coat, which directs the exit of nearly one-third of the proteins encoded by the eukaryotic genome.

In collaboration with C.S. Potter and B. Carragher, Department of Cell Biology, we have solved the 2-dimensional electron cryomicroscopy structure of the COPII cage. This cage is a self-assembling nanoparticle that collects cargo by generating a polymer scaffold. The scaffold then interacts with an adaptor platform protein complex (Sec 23/24) that links cargo to the COPII cage to generate membrane-enveloped vesicles that bud from the endoplasmic reticulum.

During formation of COPII vesicles, targeting and fusion machinery is recruited to direct the vesicle to

the next compartment, the Golgi complex. This process involves a ubiquitous family of GTPases, the Rab proteins, that direct the assembly and disassembly of tethering and fusion complexes.

Characterization of the x-ray structure of the Rab GTPase-anchored tether p115, performed in collaboration with I.A. Wilson, Department of Molecular Biology, revealed a superhelical platform that facilitates an interaction between Rab GTPases and tethering-fusion complexes to control vesicle targeting and fusion. Of high interest is our recent discovery that the ubiquitous Hsp90 chaperone system is required in the disassembly and recycling of these Rab-regulated tethering-fusion complexes and likely is responsible for maintaining the structure and function of exocytic and endocytic trafficking compartments. Moreover, using systems-level approaches, we have shown that Rab GTPases act as hubs to define the highly distinctive membrane architecture of eukaryotic cells found in different cells and tissue types. Such systems-level approaches provide a top-down global view of membrane traffic, integrating form with function.

### CONFORMATIONAL DISEASES

Proteins that do not recognize the global transport machinery governing the exocytic and endocytic pathways give rise to numerous conformational diseases that affect cellular proteostasis. For example, many mutations disrupt cargo traffic from the endoplasmic reticulum by preventing proper protein folding during synthesis, resulting in loss of recognition by the COPII machinery. In contrast, other protein conformational diseases have mutations that disrupt flow and function at later steps of the secretory pathway and outside the cell, leading to disease. In collaboration with J.W. Kelly, Department of Chemistry, we are studying the links between protein folding, trafficking defects, and the protein-folding energetics of key conformational diseases, including cystic fibrosis, Gaucher disease, type 2 diabetes, familial amyloidosis of Finnish type, other amyloidoses, Alzheimer's disease, and Parkinson's disease. A particularly important finding is that Pmel7, the major component of melanosomes, structures that form the layer in the skin that protects the body from ultraviolet light, is amyloid. Thus, amyloid, although generally considered a protein fold associated with disease, has a functional role in normal cell biology.

Our combined studies to date of conformational disease have led to a new understanding of the function of the exocytic pathway in normal host physiology,



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## Molecular Mechanisms of Olfactory Neural Circuit Formation

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K.K. Baldwin, S. Tate, B. Fields, S. Ghosh, M. Gantner, J. Hazen, A. Lu

The sense of smell influences many intriguing aspects of behavior. Scents elicit innate responses of attraction and revulsion and evoke enduring emotional memories. These features suggest that behaviorally important olfactory neural circuits may be hard-wired. We are interested in learning how genes build these neural circuits. We use gene targeting in embryonic stem cells, molecular biology, and imaging to visualize and modify the neurons that recognize odors in the periphery and the brain. By defining the specific neural circuits involved in olfaction, we hope to identify genes that regulate neural circuit formation in general. These studies are critical first steps toward understanding how neural circuits produce sensory perception and how genetic alterations may contribute to neuronal dysfunction and cognitive disorders.

### MITRAL CELLS

A major goal of our research is to understand how the distributed representation of olfactory information in the olfactory bulb is integrated in the cortex. This endeavor has been hindered by the lack of specific promoters for mitral cells, the output neurons of the olfactory bulb. We identified a gene expressed specifically in mitral cells and have engineered mice in which subsets of these cells are genetically tagged. We are tracing the projections of individual mitral cells into the olfactory cortex. We have also begun to identify genes differentially expressed in mitral cells that may regulate specific neural circuit formation.

We have shown that a large family of about 60 clustered protocadherin genes provides an unantic-

pated level of surface diversity to mitral cells. Expression of several protocadherins per neuron seems to endow each neuron with a unique cell-surface code. We have generated mice that lack protocadherin expression in defined subsets of neurons. These mice have behavioral abnormalities consistent with defects in neuronal function.

### CLONING MICE FROM NEURONS

Scientists have postulated that in addition to reversible genetic changes, irreversible chromosomal alterations occur in neurons during development, aging, and disease; however, no such alterations have been identified. The diversity and postmitotic state of neurons have hindered these studies. Currently, no method to survey the genome of postmitotic neurons exists. We are using somatic cell nuclear transfer technology to develop a method to generate cloned mice or embryonic stem cell lines from neurons. These studies will determine, for the first time, whether it is possible to clone mice from adult cortical neurons and will provide a novel means to understand how chromosomal alterations in neurons may contribute to differentiation or neurodegenerative diseases.

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## Automated Molecular Imaging

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B. Carragher, C.S. Potter, A. Cheng, D. Fellmann, C. Irving, G. Lander, P. Mercurio, S. Nayak, J. Pulokas, J. Quispe, S. Stagg, N. Vossman, C. Yoshioka

During the past decade, electron cryomicroscopy has emerged as a powerful approach for determining the structure of large macromolecular complexes. Elucidating the structure and mechanism of action of these "molecular machines" is an emerging frontier in understanding how the information in the genome is transformed into cellular activities. Examples include ribosomes, transcription complexes, track-motor complexes, and membrane-embedded pumps and channels.

In electron cryomicroscopy, the macromolecular specimen is preserved in a thin layer of vitreous (glassy) ice and imaged in an electron microscope by using low doses of electrons. The low signal-to-noise ratio of the resulting images means that averaging is required to recover the signal and reconstruct a 3-dimensional map of the structure.

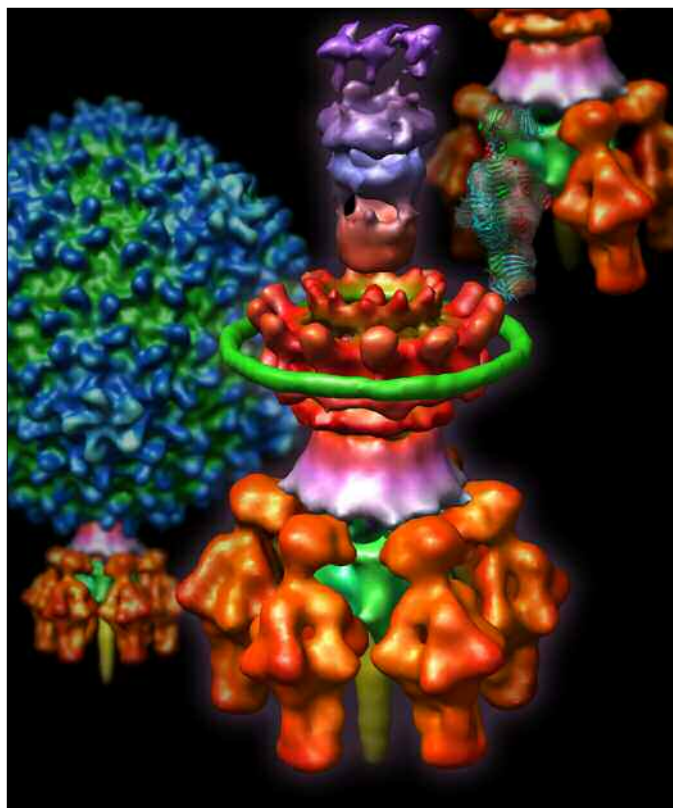
In 2002, we established the National Resource for Automated Molecular Microscopy (NRAMM) to develop,

test, and apply technology for automating the processes involved in using electron cryomicroscopy to solve macromolecular structures. The goal of automation is not only to facilitate the process of molecular microscopy, although this facilitation is a welcome benefit, but also to expand the scope of accessible problems and push experimental frontiers by making possible investigations deemed too difficult or high risk because of the considerable effort involved in using manual methods. An additional goal of automation is to enable much higher throughput of data and thus improve resolution for single-particle reconstructions by increasing the number of particles that contribute to the average 3-dimensional map. Another mission of NRAMM is to use the infrastructure developed to open up the sometimes esoteric practices of electron cryomicroscopy to a much wider group of researchers, including investigators in cell biology, x-ray crystallography, and materials science.

At NRAMM, new techniques and technologies that we have developed include new grid substrates designed to improve quality and throughput for vitreous ice specimens; a robotic grid-handling system used for screening; Leginon, an automated system for microscope control and image acquisition; a relational database that tracks and manages data acquired by Leginon and tools for viewing and delivering the data via Web browsers; and ACE, a program for the automated measurement and correction of contrast transfer function. Our current focus is the development of a fully integrated pipeline that will enable us to optimize data acquisition and processing and improve the resolution of 3-dimensional electron cryomicroscopy reconstructions.

These technological developments have been designed for and used in a number of collaborative research projects, including reconstruction of a minimal coatomer complex II cage and reconstruction of an intact infectious P22 virion (Fig. 1). The infrastructure has also, in accordance with our mission, made electron cryomicroscopy accessible to a much wider community, leading to publications from research groups in chemistry, x-ray crystallography, materials science, and industry.

NRAMM currently provides support for more than 40 collaborative and service projects each year, and the Leginon software, including the database, has been distributed to about 35 laboratories outside Scripps Research. We are also distributing ACE, a variety of other software packages, and the novel grid substrates. These efforts are complemented by training activities



**Fig. 1.** Bacteriophage P22 DNA packaging and infection machinery. Visualization of the 1.7-nm electron cryomicroscopy reconstruction of the intact bacteriophage. The portal complex (red) is hypothesized to change conformation when the virus is full of DNA, which signals the packaging motors to stop. Visualization by G. Lander. Cover for 2007 issues of *Archives of Virology*. Reprinted with permission from Springer.

that include small-group training, a biennial large training course in electron cryomicroscopy, and small workshops focused on various aspects of automation.

An additional project, sponsored by the National Science Foundation, is the development of automated data collection techniques for imaging serial sections by using an electron microscope. Understanding the fine structure of cells and cellular components contributes to a more profound understanding of cellular function and intracellular or intercellular interactions. In order to visualize these large, complex structures in 3 dimensions at resolutions sufficient to observe structure on the nanoscale, the cells must be cut into sections and then examined by using a transmission electron microscope. Acquiring high-magnification images of a long series of sections is difficult and extremely labor intensive. The region of interest in each section must be tracked across sections and across grids, a process that requires examining the sections at a variety of scales before acquiring high-magnification images of interesting areas. Multiscale imaging of this sort is

not straightforward because the image formed by an electron microscope shifts and rotates as the magnification is changed. The overall task of reconstructing a 3-dimensional volume from a set of serial sections is challenging and time consuming, and the number of large-scale reconstructions has been limited to a few spectacular examples. Our objectives are to design, develop, and implement a software application to automate the task of acquiring high-magnification images of specific regions of the cell across tens to hundreds of serial sections.

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## Chemical Physiology

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B.F. Cravatt, K. Barglow, J. Blankman, M.H. Bracey, E. Carlson, M. Dix, H. Hoover, W. Li, B. Martin, K. Masuda, A. Mulder, S. Niessen, M. McKinney, C. Salisbury, G. Simon, E. Weerapana, B. Wei

**W**e are interested in understanding complex physiology and behavior at the level of chemistry and molecules. At the center of cross talk between different physiologic processes are endogenous compounds that provide a molecular mode for intersystem communication. However, many of these molecular messages remain unknown, and even in the instances in which the participating molecules have been defined, the mechanisms by which these compounds function are for the most part still a mystery.

We are investigating a family of chemical messengers termed the fatty acid amides, which affect many physiologic functions, including sleep and pain. In particular, one member of this family, oleamide, accumulates selectively in the cerebrospinal fluid of tired animals. This finding suggests that oleamide may function as a molecular indicator of an organism's need for sleep. Another fatty acid amide, anandamide, may be an endogenous ligand for the cannabinoid receptor in the brain.

The *in vivo* levels of chemical messengers such as the fatty acid amides must be tightly regulated to maintain proper control over the influence of the messengers on brain and body physiology. We are characterizing a mechanism by which the level of fatty acid amides can be regulated *in vivo*. Fatty acid amide hydrolase degrades the fatty acid amides to inactive metabolites. Thus, the hydrolase effectively terminates the signaling messages conveyed by fatty acid amides, possibly ensuring that these molecules do not generate physiologic responses in excess of their intended purpose.

We are using transgenic and synthetic chemistry techniques to study the role of the hydrolase in the regulation of fatty acid amide levels *in vivo*. In collaboration with R.C. Stevens, Department of Molecular Biology, we solved the first 3-dimensional structure of fatty acid amide hydrolase. We are using this information to explore the molecular mechanism of action of the enzyme and to design inhibitors of the hydrolase. We are also interested in proteins responsible for the biosynthesis of fatty acid amides.

Another area of interest is the design and use of chemical probes for the global analysis of protein function. The evolving field of proteomics, defined as the simultaneous analysis of the complete protein content of given cell or tissue, encompasses considerable conceptual and technical challenges. We hope to enhance the quality of information obtained from proteomics experiments by using chemical probes that indicate the collective catalytic activities of entire classes of enzymes. These activity-based probes could be used to record variations in protein function independent of alterations in protein abundance and would be a powerful and complimentary set of tools for proteome analysis.

To date, we have succeeded in generating activity-based probes for more than a dozen enzyme classes, including serine hydrolases, metalloproteases, glutathione S-transferase, and several oxidoreductases. We are using the probes to explore the roles that enzymes play in a variety of physiologic and pathologic processes,

especially progression of cancer. We are also developing complementary strategies for profiling the complete content of metabolites in cells and tissues (the “metabolome”) to facilitate the assignment of endogenous substrates to enzymes of uncharacterized function.

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## Regulation of Cytomechanochemical Systems

G. Danuser, K. Applegate, A. Bacconi, J. Dorn, K. Jaqaman, L. Ji, J. Kunken, J. Lim, D. Loerke, M. Machacek, A. Matov, M. Sabouri, G. Yang

**W**e study how chemical signals integrate with mechanical forces to modulate cytoskeleton dynamics spatially and temporally in cell migration, cell division, and vesicular transport.

Cell migration requires accurate balancing of propulsive forces generated by assembly of actin filaments at the protruding edge of a cell, contraction forces generated by myosin motors, and adhesion forces produced in response to transmembrane linking of actin filaments to the extracellular matrix. The force balance is continually modulated by adaptation of the cytoskeleton structure in response to both local force exertion and regulatory chemical signals. Thus, understanding the interaction of diverse force-generating machinery and the structural dynamics of cytoskeleton components at a systems level is central to understanding the fundamental mechanism of cell migration.

A key aspect of cell division is the accurate segregation of replicated DNA from the mother cell into the 2 daughter cells. This segregation is achieved by the mitotic spindle, a bipolar array of microtubule polymers that symmetrically pull sister chromatids to opposite poles. Chromosome movement is mediated by forces from a large number of microtubule-associated motors and by proteins that regulate the structural dynamics of the spindle via localized microtubule assembly and disassembly. We are studying the mechanisms of force balancing during chromosome segregation at the level of the entire spindle.

We have also begun to analyze the mechanisms by which clathrin-coated structures assemble in the plasma membrane and alter the underlying cortex of actin filaments to transport molecular cargo from the extracellular domain into the cytoplasm. This so-called endocytosis is critical to all aspects of cellular homeostasis in a continually changing environment. Inside the cell, cargo packaged into vesicles is transported by motors moving along microtubules and actin cables. A special form of vesicular transport occurs in neuronal axons, where cargo is shuttled over long distances from the cell body to the peripheral regions and back. Defects in this process are known factors of neurode-

generative diseases such as Alzheimer's disease. During the past year, we set out to examine the mechanisms of local interaction between different motor types bound to an individual vesicle and how regional gradients of signals affect these interactions to ensure globally efficient transport.

To examine these molecular systems, we develop high-resolution imaging and computational methods that can be used to track the full dynamics of relevant system components in live cells. We also develop computational models to predict the relationship between the action of individual molecules in a system and the system's output at the cellular level. Currently, we are focusing on new numerical methods to relate the predictions obtained with such models to experimental data, allowing rigorous validation of the models and estimation of unknown parameters.

As one example, during the past year, we have been able to map functional groups of proteins located in kinetochores, a dense complex of 70–100 different proteins that mediates chromosome-microtubule attachment in the mitotic spindle. By combining yeast genetics, fully automated imaging, and image analysis of individual chromosome movements, we distinguished motion phenotypes that revealed functional interactions between components of the kinetochore. To our knowledge, these results are the first to indicate that molecular interactions can be identified on the basis of the similarity of effects several genes have on the dynamics of a specific molecular machine. Using this approach, we will systematically probe the pathways that transform force and chemical signals across the kinetochores into microtubule dynamics and chromosome movement. More generally, our results suggest a new model for the functional analysis of multiprotein complexes that are too large for biochemical and structural investigation.

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## Synthetic Protein Chemistry

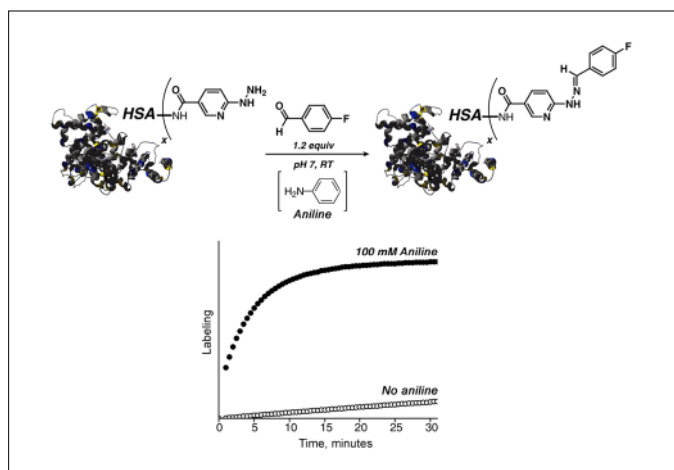
P.E. Dawson, J.B. Blanco-Canosa, F. Brunel, M. Churchill, A. Dirksen, E. Lempens, N. Metanis, C. Schroeder, T. Shekhter, T. Tiefenbrunn

**W**e use synthetic chemistry to construct macromolecules of biological importance. We have developed a set of highly selective chemical reactions that allow unprotected peptides, proteins, nucleic acids, or fragments of carbohydrate to be assembled selectively into functional macromolecules. For example, using these methods, we can incorporate unnatural amino acids to probe fundamental questions about protein folding, stability, and enzymatic catalysis. In addition, we can specifically label proteins with agents for fluorescent imaging, positron emission tomography, or magnetic resonance imaging. We used these methods to incorporate specific deuterium probes for the vibrational analysis of proteins by infrared spectroscopy in studies with F.E. Romesberg, Department of Chemistry, and to transfer fluorescent labels to specific exosites on thrombin in studies with colleagues in the Department of Molecular and Experimental Medicine. We are also part of a collaborative project to use diverse techniques, including synthetic protein chemistry, to mimic HIV epitopes; the goal is to develop HIV vaccines.

The ability to chemically modify biological macromolecules in a specific manner underlies many of the

methods and technologies used in modern research. This specific tailoring has been enabled by the development of highly chemoselective ligation (conjugation) chemistries that are characterized by their chemoselectivity, reactivity, and compatibility with neutral aqueous buffers. However, the growing demands of research in analytical biochemistry, chemical biology, protein chemistry, and nanotechnology have pushed the limits of currently available ligation methods, especially in terms of ligation kinetics.

This past year, we showed that the aromatic amine aniline is a potent nucleophilic catalyst for imine ligations that form stable oximes and hydrazones from aldehyde and amine-labeled precursors (Fig. 1). We



**Fig. 1.** The small molecule aniline catalyzes hydrazone reactions used for biomolecular labeling. HSA = human serum albumin.

have used this catalyst to optimize imine reactions to attain rates greater than  $1000 \text{ M}^{-1} \text{ s}^{-1}$ , several times faster than generally used ligation approaches. Such fast conjugation rates are essential if chemical approaches are ever to compete with the rapid labeling possible via noncovalent interactions with agents such as biotin or antibodies. Importantly, because the ligation rate is determined by the amount of catalyst, we can tune the reaction rate to fit a desired application.

We are using this chemistry to efficiently label proteins such as RANTES, albumin, myoglobin, and annexin A5 with materials for fluorescence imaging, positron emission tomography, and magnetic resonance imaging and to label nanoparticles. Future research will be on using aniline as a catalyst in complex biological systems to label cell-surface and intracellular components and to generate protein and carbohydrate arrays.

In addition to providing rapid and selective reaction rates, this catalytic approach enables the reversible labeling of molecules. Using rapid reversible

covalent chemistry, we anticipate being able to select molecules on the basis of binding affinity or target selectivity. We think that these reactions will enable us to develop reversible tagging strategies compatible with complex biological systems.

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## Regulation of Actin Dynamics in Morphogenesis and Development

V.M. Fowler, R.S. Fischer, R.A. Lewis, C. McKeown, R. Nowak, K.L. Weber

**R**egulation of actin dynamics at the ends of filaments determines the organization and turnover of actin cytoskeletal structures and is crucial for cell motility and architecture and for actin-based morphogenetic processes in development and tissue function. We focus on tropomodulins, proteins that block actin association and dissociation at the pointed ends of filaments, stabilizing the actin filaments and regulating their length. Vertebrates have 4 tropomodulin isoforms; each isoform is about 40 kD and has a homolog in flies and worms. Unique among all actin-capping proteins, tropomodulins also bind to tropomyosin to tightly cap tropomyosin-coated actin filaments with affinities more than 1000-fold greater than the affinities for pure actin filaments. Disassembly of actin at the pointed ends of filaments is the rate-limiting step in the turnover of actin filaments, yet relatively little is known about the regulation of pointed ends. Tropomodulins are the only capping proteins of pointed ends

and thus are expected to have critical roles in regulation of actin turnover and stability in actin-based morphogenetic processes. Previously, we showed that tropomodulin 1 regulation of the length and stability of thin filaments is essential for the assembly and development of cardiac myofibrils *in vivo*; mouse embryos without tropomodulin 1 die at 9.5 days of development with a malformed and nonfunctional heart tube.

Tropomodulins have 2 domains: an unstructured, flexible N-terminal domain and a compact, folded C-terminal domain consisting of 5 leucine-rich repeats and a terminal  $\alpha$ -helix. The N-terminal domain binds tropomyosin and caps tropomyosin-actin pointed ends with nanomolar affinity; the C-terminal domain caps actin pointed ends with submicromolar affinity and is unaffected by tropomyosin. Despite the high level of sequence conservation (about 70%) among vertebrate tropomodulins, tropomodulin 3, but not tropomodulin 1, also binds actin monomers in addition to capping pointed ends and binding tropomyosin. This novel monomer binding activity may be critical for control of actin assembly and turnover in dynamic actin filament networks by tropomodulin 3 in the lamellipodia of crawling endothelial cells, where this tropomodulin functions as a negative regulator of cell migration. Tropomodulin isoforms can also be distinguished by their different binding affinities for the many tropomyosin isoforms produced by alternative splicing from 4 genes. We are using site-directed mutagenesis to define the amino acid residues that confer diverse tropomyosin and actin regulatory properties upon different tropomodulin isoforms; our goal is to develop mutants for studies of cellular functions.

During the past year, we focused on the functions of tropomodulins in polarized epithelial cells. We used the colon intestinal epithelial cell line Caco2, which forms well-polarized monolayers containing tropomodulin 3 in the actin filament cortex at the lateral membranes. We found that reduction of tropomodulin 3 levels by small hairpin RNA led to a significant loss of filamentous actin from lateral cell membranes and a decrease in epithelial cell height along with a corresponding increase in cell-surface area. These morphologic changes took place without loss of cell polarity, as indicated by unchanged localization of protein at tight junctions and adherens junctions. Strikingly, tropomyosins are lost from the cortical actin cytoskeleton when tropomodulin 3 levels are decreased, suggesting that loss of tropomodulin 3 leads to dissociation of tropomyosin followed by disassembly of the cortical actin filaments. Actin

filaments stabilized by tropomodulin 3 and tropomyosin may contribute to cell height by providing actin filaments for myosin contraction or by promoting assembly of the spectrin-based membrane skeleton, which may both contribute to epithelial cell shape and height.

The eye lens is a specialized epithelial tissue composed of radially arranged layers of long, thin fiber cells tightly packed in a regular hexagonal pattern that is essential for lens transparency and optical function. Both tropomodulin 1 and tropomodulin 3 are present on the lateral membranes and in the cytoplasm of lens fiber cells in mice. To investigate the function of tropomodulin 1 in the lens *in vivo*, we used transgenic mice that had no tropomodulin 1 in the lens.

In the transgenic mice, the lens develops normally in the absence of tropomodulin 1, but as the lens grows after birth, the regular hexagonal packing organization of the fiber cells becomes disordered and the cross-sectional shapes and sizes of the cells are abnormal. Levels of a membrane-associated short tropomyosin isoform are reduced dramatically, and a subpopulation of actin filaments on the lateral membranes disassembles and polymerizes in the cytoplasm of the fiber cells. Actin filaments stabilized by tropomodulin 1 and tropomyosin may be required to generate tensile forces on fiber cell lateral membranes that maintain the uniform shape of fiber cells and hexagonal packing symmetry as the lens increases in size. We are developing mice that lack the gene for tropomodulin 3 to assess the effect of a complete lack of all tropomodulins in epithelial morphogenesis and function *in vivo*.

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## Nucleocytoplasmic Transport and Signal Transduction in the Nucleus

L. Gerace, G. Ambrus-Aikelin, A. Cassany, B. Chen, E. Choi, K. Datta, T. Guan, M. Huber

The functional activities of the eukaryotic nucleus are spatially and temporally separated from the cytoplasm by the nuclear envelope, a specialized subdomain of the endoplasmic reticulum. Trafficking of proteins and RNAs across the nuclear envelope is accomplished by nuclear pore complexes (NPCs), which provide gated channels for receptor-mediated transport. In addition to providing a barrier that separates the nucleus from the cytoplasm, the nuclear envelope has an important role in signal transduction in the nucleus. Many signaling functions of the nuclear envelope are associated with the nuclear lamina, a protein meshwork lining the inner nuclear membrane that additionally provides a structural scaffold for the nucleus and chromosomes. Over 15 human genetic disorders are due to mutations in lamina components, underscoring the importance of the lamina in nuclear structure and function. We are studying the molecular mechanisms of nucleocytoplasmic trafficking and the role of the lamina in signaling and nuclear organization, particularly as related to muscular dystrophies and other human diseases.

### NUCLEOCYTOPLASMIC TRANSPORT MECHANISMS

Transport of protein and RNA through NPCs is an energy-dependent process mediated by nucleocytoplasmic shuttling receptors of the karyopherin  $\beta$  family. Karyopherins bind to transport signals on protein or RNA cargo molecules, and the receptor-cargo complexes are translocated through the NPC by receptor binding to a group of NPC proteins (nucleoporins) that contain phenylalanine-glycine amino acid motifs. The directionality of nuclear transport is determined largely by the small GTPase Ran, which directly interacts with karyopherins and thereby regulates cargo binding. Conformational flexibility of karyopherins is thought to be fundamental to their dynamic interactions with cargo, Ran, and nucleoporins.

We are using *in vitro* assays with digitonin-permeabilized cells to analyze the molecular events that specify translocation of cargo-receptor complexes through NPCs. Recently, using site-directed mutagenesis of importin  $\beta$ , the prototypical nuclear import receptor, we

characterized 2 distinct binding sites in importin  $\beta$  for nucleoporins containing the phenylalanine-glycine motif and defined mutational hot spots for cargo binding. A major goal is to determine how the conformational dynamics of importin  $\beta$  are linked to discrete transport steps. To this end, we are complementing structure-function studies with analysis involving small-molecule inhibitors.

In a related project, we are analyzing nuclear import of the adenovirus genome, which consists of a 36-kb double-stranded DNA molecule. Results from our *in vitro* transport studies indicate that transport of adenovirus DNA is driven by import signals on DNA-associated proteins. Our characterization of multiple import signals in adenovirus protein VII and the tight association of the protein with the genome suggest that this viral protein may be the protein adaptor involved in the DNA import. Nuclear import of protein VII involves several of the major cellular importins, suggesting that adenovirus has evolved to use redundant import pathways to ensure efficient nuclear delivery of its genome. We have also found that the nucleoporin Nup214 provides a docking site at the NPC for the adenoviral nucleocapsid and plays a key functional role in adenovirus DNA import.

We are also analyzing nuclear export of unspliced HIV type 1 mRNA, which requires the viral regulatory protein Rev. Rev polymerizes on a *cis*-acting sequence of viral mRNAs, termed the Rev response element, providing a platform for assembly of nuclear export factors. In collaboration with J.R. Yates, Department of Cell Biology, we used proteomics analysis of nuclear extracts to characterize the proteins assembled on the complex formed by Rev and the Rev response element. The proteins we have identified may determine the epigenetic fate of HIV type 1 transcripts and, accordingly, could be targets for new therapeutic drugs.

### NUCLEAR LAMINA AND INTRANUCLEAR SIGNAL TRANSDUCTION

The nuclear lamina contains a polymer of 2–4 related intermediate filament proteins called lamins, which are associated with a number of transmembrane proteins of the inner nuclear membrane. In collaboration with Dr. Yates, we identified 67 novel nuclear envelope-enriched transmembrane proteins in nuclear envelopes from rodent liver, indicating that the lamin proteome is much more extensive than previously thought. We are focusing on analysis of these proteins in muscle, because muscle is the tissue most sensitive

to disruption of lamina function by disease-causing mutations. Using transcriptional profiling of cultured myoblasts, we found that the genes of 6 the transmembrane proteins are strongly upregulated in myoblast differentiation and also are highly expressed in muscle in adults, consistent with a role of the genes in muscle differentiation and/or maintenance. The 3 members of this group of genes that we have analyzed so far by using RNA interference are all required for myoblast differentiation. Interestingly, on the basis of their sequence homologies, these nuclear envelope-enriched transmembrane proteins are predicted to have roles in intranuclear signaling. Our results suggest that these and other signaling functions of the nuclear envelope may be perturbed in various human diseases associated with mutations in components of the lamina.

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## Organization and Function of the Neuronal Cytoskeleton

S. Halpain, B. Calabrese, L. Dehmelt, E. Hwang,  
B. Jenkins, K. Spencer, G. Poplawski

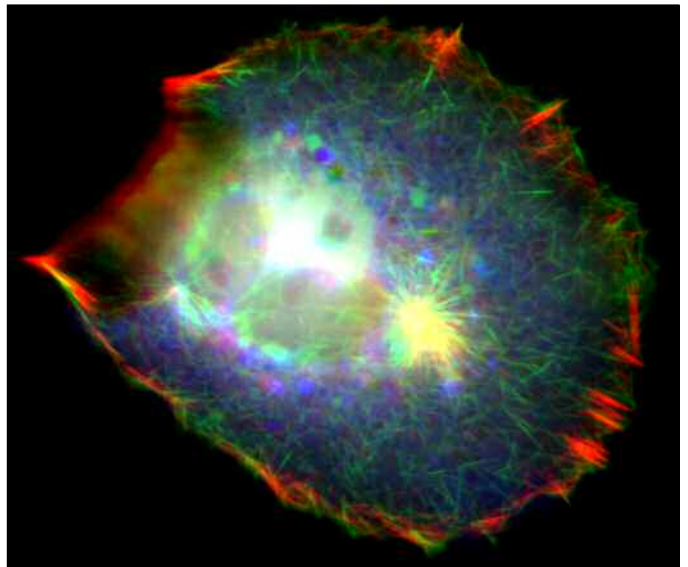
**D**uring the past year, we made significant progress in research on the development and regeneration of neurons. In 2 main projects, we focused on cytoskeletal proteins of nerve cells, key proteins that underlie the structure and morphologic flexibility required by neurons for transmitting, storing, and processing synaptic signals. We used biochemical, molecular biological, and microscopy-based approaches to understand the function of these molecules. Fluorescence time-lapse imaging of living neurons is an important tool that we used to uncover structure-function relationships for cytoskeletal proteins and the consequences of the dysfunction of the proteins. The results of these projects have contributed to our understanding of molecular events in normal brain development and in regeneration of neuronal structure after injury and disease.

#### MICROTUBULE-ASSOCIATED PROTEINS

One project concerns microtubule-associated proteins (MAPs). These proteins are important in regulating the assembly and stability of microtubules and the

interactions of microtubules with other components of the cytoskeleton. We found that one microtubule-binding protein, MAP2, also directly binds actin filaments and induces filament bundling. Using fluorescence-based time-lapse imaging and high-resolution confocal microscopy, we tracked the behaviors of microtubules and actin filaments in living neuronal cells with normal and mutant forms of MAP2.

Recently, we found that the microtubule-based molecular motor dynein plays a key role in transporting microtubules toward the cell periphery (Fig. 1).



**Fig. 1.** Time collage of MAP2c-induced reorganization of microtubules. Microtubules are depolymerized by nocodazole (time 00:00 shown in blue). Shortly after drug washout (green), microtubule bundles travel rapidly through the cell and subsequently (red) accumulate at the cell periphery through the actions of the molecular motor dynein. Dynein activity is essential for neurite initiation. Image courtesy of L. Dehmelt.

This dynein-dependent activity provides a key force that pushes the cell membrane outward during neurite initiation. Currently, we are using proteomic approaches and high-content, microscopy-based screening technology to identify other cytoskeletal proteins and signal transduction pathways crucial to the initiation of neurites.

#### DENDRITIC SPINES

A second project concerns the regulation of dendritic spines, specialized cellular protrusions that form the receptive, postsynaptic element at glutamate synapses. Spines become lost or dysmorphic in many types of mental retardation and in psychiatric conditions such as chronic depression and schizophrenia. Furthermore, spines are vulnerable to injury in diseases such as stroke and epilepsy, in which excessive release of glutamate can induce neuronal injury and subsequent cell death

(a condition termed excitotoxicity). Understanding how spines form, what regulates their stability, and how they recover from injury is therefore of therapeutic interest for several neurologic conditions.

Recently, we found that amyloid  $\beta$ -peptide, a key molecule directly associated with mechanisms in the pathologic changes associated with Alzheimer's disease, can profoundly alter the morphologic stability of synapses. We found that picomolar concentrations of a natural form of amyloid  $\beta$ -peptide (comparable to the levels found in the cerebral spinal fluid of patients with Alzheimer's disease) induced rapid changes, within 1–2 hours, in glutamatergic synapses of cultured hippocampal neurons. We found significant decreases in clusters of the presynaptic protein synaptophysin and concurrent decreases in the numbers of dendritic spines. Remaining spines usually became abnormal in shape. Time-lapse imaging showed that both presynaptic and postsynaptic injury occurred at single synapses and that the effects were reversed upon removal of amyloid  $\beta$ -peptide. Electrophysiologic data confirmed these synaptic changes.

In addition, we found that specific inhibitors of either nicotinic acetylcholine receptors or *N*-methyl-D-aspartate glutamate receptors would prevent these spine changes, providing an important link between our *in vitro* studies and published clinical findings, because blockers of these receptors are used as front-line drugs in the treatment of Alzheimer's disease. Together our studies suggest that low levels of amyloid  $\beta$ -peptide have deleterious effects at individual synapses and that these effects may be partly or wholly responsible for the progressive loss in cognition and memory that characterizes the early stages of Alzheimer's disease. Our results also point toward new potential molecular targets in drug development to treat Alzheimer's disease.

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## Function of Nuclear Receptors in Stress and Mitochondrial Homeostasis

A. Kralli, B. Hazen, M.B. Hock, K. Machini, C. Tiraby-Nguyen, J.A. Villena

**W**e are interested in the molecular mechanisms that relay metabolic stress signals to a network of transcriptional regulators and the ensuing transcriptional outputs that mediate adaptive metabolic responses to the stress signals. In particular, we focus on the coactivators peroxisome proliferator-activated receptor  $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) and PGC-1 $\beta$  and the orphan nuclear receptors of the estrogen-related receptor (ERR) subfamily, which control mitochondrial biogenesis and energy homeostasis. Our goals are to elucidate the biology of this transcriptional network in skeletal muscle and the CNS, understand how deregulation of the network leads to disease, and ultimately identify the components of the network that are most suitable for drug intervention to counteract metabolic disease.

#### REGULATION OF THE PGC-1/ERR NETWORK

Levels of expression and activity of PGC-1 $\alpha$  and PGC-1 $\beta$  are regulated by signals that relay changes in metabolic needs. The coactivators then transmit such signals, via interactions with ERRs and other transcription factors, to regulate expression of target genes that mediate adaptation to the new energetic needs. We are interested in the mechanisms that regulate PGC-1s at the posttranslational level via covalent modifications or via interaction with other proteins and thereby control the properties of the PGC-1/ERR network. In collaboration with M. Stallcup, University of Southern California, we showed that PGC-1 $\alpha$  is methylated by the protein arginine methyltransferase 1 and that this methylation increases the activity of the coactivator and leads to the enhanced expression of genes with roles in mitochondrial function. Currently, we are investigating the molecular mechanisms by which methylation regulates PGC-1 $\alpha$  activity. Finally, in collaboration with S.I. Reed, Department of Molecular Biology, we are elucidating posttranslational modifications that regulate the stability of PGC-1 $\alpha$ .

### ROLE OF THE PGC-1/ERR NETWORK IN MITOCHONDRIAL FUNCTION AND MUSCLE PHYSIOLOGY

Our previous cell culture studies suggested that the effects of PGC-1 $\alpha$  and PGC-1 $\beta$  on mitochondrial biogenesis and function are mediated primarily by the orphan nuclear receptor ERR $\alpha$ . In confirmation of such a role for ERR $\alpha$ , we have shown that the brown adipose tissue of mice that lack ERR $\alpha$  has decreased levels of mitochondria, decreased oxidative capacity, and increased lipid deposits. These mice cannot maintain their body temperature when exposed to cold because of a deficit in the generation of energy required for thermogenesis. These findings established for the first time that ERR $\alpha$  *in vivo* is an important component of the regulatory network that promotes mitochondrial biogenesis and that this receptor is essential under conditions of energetic stress (e.g., exposure to cold). Currently, we are focusing on the physiologic significance of decreased ERR $\alpha$  function in skeletal muscle and the possible involvement of the receptor in metabolic adaptation to physical exercise.

The family of ERRs includes 2 additional receptors, ERR $\beta$  and ERR $\gamma$ , whose physiologic roles are not yet clear. Recent studies suggest that ERR $\beta$  and ERR $\gamma$  may also regulate genes with roles in energy homeostasis and thereby compensate, at least partially, for the lack of ERR $\alpha$  in mice that lack the genes for ERR $\alpha$ . To determine the specific roles and relative contributions of these factors in adaptive metabolic responses, we use molecular genetics and biochemical approaches in cell culture and mouse model systems. Mitochondrial dysfunction and deregulation of energy homeostasis in skeletal muscle have been implicated as underlying causes of insulin resistance and type 2 diabetes and as contributing factors in muscle degenerative diseases. We use mouse models in which the animals lack ERR $\alpha$ , ERR $\beta$ , and/or ERR $\gamma$  specifically in skeletal muscle to elucidate the roles of ERRs in skeletal muscle physiology and metabolism, at basal levels and upon metabolic challenges (e.g., physical exercise, muscle injury, caloric overload).

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## Structural and Functional Proteomics for Novel Therapeutics and Diagnostics

P. Kuhn, J. Nieva,\* E. Abola, A. Brooun, R. Bruce, J. Chrencik, P. Clark, S. Coon, S. Daudenarde, D. Davis, J. Dupuy, S. Foster, J. Joseph, A. Kolatkar, N. Lazarus, M. Leach, D. Marrinucci, K. Saikatendu, V. Subramanian, M. Yadav, B. Wu, Q. Zhao, Z. Qiang

\* Department of Molecular and Experimental Medicine, Scripps Research

#### DETECTING RARE CELLS IN CIRCULATION

Many clinically important cells in blood occur at frequencies of less than 1 per 1 million cells. Detecting and characterizing these rare cells require the development of new technologies that operate with exceptional specificity. Scientists at the Scripps-PARC Institute for Advanced Biomedical Sciences have developed an instrument, based on fiber-optic array scanning technology, that provides rapid and accurate identification of rare cells in the circulation in humans. Potential clinical applications include detecting circulating tumor cells, circulating endothelial cells, and fetal cells in the maternal circulation.

Malignant cells from solid tumors begin to circulate at the earliest stages in cancer formation. The circulating cells are quite rare, occurring at a frequency of less than 1 malignant cell for every 1 million normal nucleated blood cells. Technology to detect and characterize these cells can be valuable in screening for cancer and in guiding individualized cancer therapy.

#### STRUCTURAL PROTEOMICS AND DRUG DISCOVERY

The goal of our research on protein-protein interactions is a thorough analysis of the structural, biophysical, dynamic, and chemical foundations of these interactions. The signaling cascades of the Eph receptor tyrosine kinase family and the ephrin ligands have been the cornerstone of this research. Although investigations initially focused on the Eph-ephrin interaction in axonal guidance, cell attachment, and motility, now evidence indicates this interaction is dysregulated in

numerous cancerous tissues. We have solved the crystal structure of the EphB2 receptor in complex with an antagonistic peptide that inhibits tumor progression *in vivo*. This structure reveals novel features that should help in the development of compounds that effectively modulate Eph-ephrin interactions and biological activities. On the basis of the accumulating structural information on Eph receptors, we have developed high-throughput screening to identify novel compounds that interfere with Eph-ephrin signaling and therefore tumorigenesis.

Members of the Dbl family of guanine nucleotide exchange factors play important roles in mediating vital cellular processes such as cytoskeleton rearrangements, mitogenesis, transcriptional changes, lymphocyte activation, and malignant transformation by interacting with Rho GTPases. Inhibition of the Vav1-Rac1 interaction is an attractive target for immunosuppression. We have determined the structure of the Vav1-Rac1 complex and have defined novel features of this interaction that should accelerate drug discovery efforts in transplantation and cancer.

#### **STRUCTURAL AND FUNCTIONAL PROTEOMICS ANALYSIS OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS**

We are generating a structure-function-interaction map of the severe acute respiratory syndrome coronavirus (SARS-CoV) proteome and the interactions of the virus with the host cell to define a comprehensive set of targets for rational, structure-based drug and vaccine design. We use bioinformatics, structural biology, genetic methods, and functional assays.

So far, we have determined the structures of 9 SARS-CoV proteins. We used crystallography for 5 non-structural proteins, nuclear magnetic resonance for 3 other nonstructural proteins, and electron cryomicroscopy for a large surface glycoprotein spike. These studies are providing important information on formation of the replicase complex, transcription, and RNA-processing events unique to the SARS-CoV life cycle. A total of 10 other protein domains have been successfully expressed in soluble, folded forms; their structures are being determined. In addition, we have begun high-throughput fragment-based nanocalorimetry studies of SARS-CoV enzymes to identify lead compounds for effective antiviral agents in SARS.

#### **ACCELERATED TECHNOLOGIES CENTER FOR GENE TO 3D STRUCTURE**

Scientists at the Accelerated Technologies Center for Gene to 3D Structure are simultaneously develop-

ing, operating, and deploying 3 key technologies to improve costs of using x-ray crystallography to determine the structure of experimental proteins. The first technology is computer-aided design of expression-optimized synthetic genes and protein constructs for crystallography. Cloning via gene synthesis improves the success rate for gene isolation and allows researchers to engineer the gene sequence of interest to be optimized for protein production in a desired heterologous expression system. The second technology, microfluidic plug-based nanovolume protein crystallization in microcapillaries for *in situ* x-ray screening and data collection, is economical and greatly broadens the range of useful quantities of proteins required for crystal growth. This technology also allows for fine control over chemical gradients in crystal growth, thereby expanding the coverage of crystallization space without consuming large quantities of protein.

The third technology, the compact light source, is a tunable laboratory x-ray source with peak intensity at x-ray wavelengths that span selenium anomalous absorbance. Having a tunable laboratory x-ray source and a crystal inventory in the same facility will greatly enhance the ability to efficiently solve new protein crystal structures.

The future integration of such technologies in a single facility at Scripps Research will enable efficient gene design for improved protein production, small-volume crystallization with *in situ* x-ray diffraction screening, and tunable x-ray data collection in a single laboratory.

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# Molecular Imaging and Tumor Targeting With Virus-Based Nanoparticles

M. Manchester, G. Destito, M. Estrada, K. Koudelka, C. Rae, P. Singh, D. Thomas

**C**urrent treatment of cancer typically involves chemotherapies that have severe adverse effects. The requirement that patients must withstand the toxic effects of treatment often limits the effectiveness of the therapy. Further, many promising anticancer compounds that are highly effective *in vitro* are too toxic to be used *in vivo*.

The ability to specifically target therapies to the site of a developing tumor while avoiding healthy tissue is an important goal for cancer research. Similarly, a tremendous need exists to identify, image, and monitor tumors, particularly at early stages and during treatment. Recently, "smart" nanoparticles, which combine these multiple targeting, imaging, and drug delivery functions, have been developed. Therapies based on nanoparticles have tremendous potential to increase the sensitivity and specificity of diagnostic imaging and treatment. Many different classes of nanoparticles are currently in development, including dendrimers, liposomes, paramagnetic nanoparticles, and quantum dots. We focus on virus-based nanoparticles as platforms for the development of tissue-specific targeting and imaging agents *in vivo*, primarily focusing on cowpea mosaic virus (CPMV).

## CPMV AND SENSITIVE *IN VIVO* IMAGING OF MAMMALIAN CELLS

CPMV is an icosahedral, 3-nm particle that is produced easily and inexpensively in black-eyed pea plants. In contrast to the structure of most other nanomaterials, the structure of the CPMV capsid is defined and can be engineered to display peptides or proteins in controlled orientations on particle surfaces via either genetic manipulation of the viral genome or by chemical attachment to the particle surface. CPMV is bioavailable and non-toxic, and the capsids are highly stable to temperature, pH, and the conditions required for chemical reactions.

By conjugation to surface lysine residues, CPMV can be labeled with fluorophores at high densities, resulting in an extremely bright, nontoxic material that is an outstanding tool for imaging vasculature in live animals. We previously showed that CPMV can be used to effec-

tively image the complete vasculature in the embryos of several species and that it is superior to other imaging particles such as lectins, fluorescent dextrans, or polystyrene microspheres.

CPMV particles have also been highly useful in highlighting angiogenesis in developing tumors. Uptake of particles into endothelial cells occurs, yielding a bright imaging signal that can be used to differentiate between arterial and venous vessels. This characteristic is rather surprising because CPMV infects plants, not vertebrates. Nevertheless, we recently showed that endothelial uptake is mediated by a 53-kD cellular membrane protein. Proteomic identification of this protein and its role in endothelial cell and tumor cell architecture are being determined.

## TUMOR TARGETING WITH VIRUS-BASED NANOPARTICLES

We have designed virus-based nanoparticles that are specifically targeted to tumors *in vivo*. Bioconjugation of CPMV to tumor ligands, whose receptors are upregulated on metabolically active tumor cells, facilitates specific particle-tumor interactions. Further, we characterized the *in vivo* pharmacokinetics and toxicity of natural and surface-modified CPMV particles. Finally, preliminary studies indicate that the cytotoxic effects of chemotherapeutic agents are enhanced when the agents are delivered to tumors inside virus-based nanoparticles.

These studies will allow the further design of antitumor agents that can provide localized, highly specific imaging and therapy *in vivo*. Use of virus-based nanoparticles may help us visualize and eliminate small tumors before the tumors can metastasize. In addition, the ability of the particles to focus toxic effects to the site of the malignant cells, thereby expanding the range of effective therapies that can be used *in vivo*, holds great promise for reducing cancer-related morbidity and mortality.

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## Translational Regulation in Chloroplasts and Expression of Human Therapeutic Proteins in Eukaryotic Algae

S.P. Mayfield, A. Manuell, M. Beligni, J. Marín-Navarro, M. Muto, M. Tran, D. Siefker, S. Chen, J. Wu

Understanding gene expression in chloroplasts and how this process is regulated is key to understanding plant development and function. Eukaryotic algae are an excellent system for investigating chloroplast function and are also an ideal system for biotechnological applications, including the production of human therapeutic proteins. Controlling gene expression in chloroplasts is essential for optimizing the production of human therapeutic proteins, and understanding translation in chloroplasts is essential for understanding gene expression in these organelles.

The core translational apparatus of chloroplasts is related to that of bacteria, but chloroplasts have incorporated novel protein components that allow for more complex regulatory mechanisms. Some of these components are ribosomal proteins; others are protein translation factors. Chloroplast mRNAs also contain unique RNA regulatory elements that are not found in bacteria, as well as conserved RNA elements such as Shine-Dalgarno sequences, but even these conserved elements appear to function differently in chloroplast translation than in bacterial translation.

To better understand translation in chloroplasts, we are characterizing the structures of both the chloroplast and cytoplasmic ribosomes from *Chlamydomonas reinhardtii*, a unicellular photosynthetic alga. Using electron cryomicroscopy and single-particle reconstruction, we determined the structure of the *C. reinhardtii* cytoplasmic 80S ribosome and found that it is nearly identical to 80S ribosomes from animals, including those of mammals. We also determined the structure of the chloroplast ribosome to 15 Å and found that although it is conserved with bacterial 70S ribosomes, it has large unique structural domains, as predicted by our proteomic analysis.

The unique structural domains of the chloroplast ribosome are located primarily on the small ribosomal subunit. This finding is supported by proteomic analysis that indicated that the mass of the small (30S) sub-

unit of the chloroplast ribosome is 25% larger than the bacterial 30S subunit. Chloroplast-unique structures are found on the solvent side of the small subunit; the large subunit is similar to that in bacterial ribosomes. The largest of the chloroplast-unique domains occur in the vicinity of the mRNA entrance and exit tunnels and extend across the entire solvent-exposed face of the chloroplast small ribosomal subunit. These chloroplast-unique ribosomal structures are poised to interact with chloroplast mRNAs early in message recognition, a key point for translational regulation.

These studies have revealed the structural basis from which we can identify the molecular and biochemical interactions of mRNAs, translation factors, and ribosomes that result in regulated translation of chloroplast mRNAs.

Chloroplast translation is light activated and involves light-induced redox changes, alterations in the ATP/ADP levels, and the generation of a proton gradient across the thylakoid membrane. The specific molecular mechanisms that allow these biochemical changes to influence translation are not completely understood. Our current model for light-activated translation proposes that reducing equivalents generated by light through photosynthetic electron transport are propagated in a cascade of redox reactions to thioredoxin, which then activates the binding of a protein complex to plastid mRNAs. Binding of this protein complex allows ribosome association of the mRNA and hence increased translation. Light-induced translation activation requires only the 5' untranslated region of mRNAs and photosystem I, presumably for the generation of reducing potential. Experiments with a proton uncoupler showed that a proton gradient across the thylakoid membrane is also required for light-activated translation, although how this gradient is used to activate translation is unclear.

Chloroplast translation can also be regulated by autoattenuation, in which an excess of unassembled protein represses translation of the proteins' own mRNA. Light activation can overcome autoattenuation, suggesting that autoattenuation may function through the same RNA-binding proteins identified as components of light-activated translation. How the proteins involved in light activation interact with the unique chloroplast ribosomal proteins is completely unknown, and understanding these interactions will be an important aspect of our research in the coming years.

In addition to these basic studies on translation, we have developed a system for the expression of

recombinant proteins, including human therapeutic proteins, in *C reinhardtii* chloroplasts. We have expressed a number of mammalian proteins, including monoclonal antibodies and mammalian growth factors, and have shown that this alga-based system can produce human therapeutic proteins at high levels. Most recently, we have focused on producing fusion proteins, in which a targeting antibody domain is linked to a cell-killing toxin. Using this technology, we have produced an antibody-toxin fusion protein that binds and kills human B-cell lymphomas, and cell-based assays have shown the efficacy and specificity of this molecule. These proteins have great potential for the treatment of cancers and infectious diseases, and chloroplasts offer perhaps the only system in which these types of proteins can be produced.

These studies have shown the tremendous potential of eukaryotic algae for the expression of recombinant human therapeutic proteins, because algae can be grown economically at large scale. Our continued genetic, biochemical, and structural studies should lead to a greater understanding of the mechanism of chloroplast translation and enable us to design appropriate transgenes to affect higher levels of expression of therapeutic proteins.

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## Molecular Basis of Cognitive Function and Dysfunction

M. Mayford, G.J. Reijmers, M. Yasuda, N. Matsuo

**T**he ability to remember is perhaps the most significant and distinctive feature of our cognitive life. We are who we are in large part because of what we have learned and what we remember. Impairments in learning and memory are a component of disorders that affect human beings throughout life, from

childhood forms of mental retardation to psychiatric disorders such as schizophrenia with onsets in late adolescence and early adulthood to diseases of aging such as Alzheimer's. We use genetic manipulation in mice to investigate the molecular events involved in learning and memory.

#### CALCIUM SIGNALING AND MEMORY

We know relatively little at a molecular level about how the brain stores new information. One hypothesis, which we have tested, is that calcium-regulated changes in the strength of synaptic connections between nerve cells can store information. The calcium/calmodulin-dependent protein kinase is abundant at synapses and when activated by calcium can strengthen synaptic connections. We used genetic manipulations in mice to indiscriminately activate this kinase at all synapses in the entorhinal cortex, a part of the brain important for memory that is affected in the earliest stages of Alzheimer's disease in humans.

We found not only that the formation of new memories is impaired but also that previously established memories could be erased. If memories are stored as precise patterns of synaptic weights, then the indiscriminate strengthening of synapses might be expected to erase memories in a manner similar to the way writing all 1's in computer memory will erase previously stored information. We also examined where calcium/calmodulin-dependent protein kinase functions within cells. We found that the synthesis of this kinase from RNA located specifically at synapses is necessary for the stabilization of memories that last several months.

#### MOLECULAR ANATOMY OF MEMORY

When humans learn new information, they use only a tiny fraction of the neurons in the brain. One difficulty in studying memory is an inability to identify and specifically manipulate those neurons that participate in a particular memory trace. We developed a genetic technique for use in mice that enables us to specifically introduce genetic changes into neurons that are activated by behavioral stimuli. By introducing a visible marker protein, we can permanently tag activated subsets of neurons, creating a precise record of the activity pattern at a specific time. We used this approach to address a basic unanswered question in neuroscience: Do the same neurons that are activated during learning become reactivated during recall of the memory? Using fear conditioning in mice, a behavioral model of post-traumatic stress disorder, we found that the same neurons activated during learning were reactivated when

an animal recalled the fearful event. We also used this approach to study extinction, a process used in the treatment of phobias by which memories are weakened by repeated exposure to a relevant stimulus. We found the neurons originally activated by a fearful stimulus were no longer activated after extinction. This finding suggests that extinction training actually erases or interferes with some component of the original memory trace.

#### FREE RADICALS IN AGING AND DISEASE

Cellular damage caused by free radicals is thought to be a component of many diseases of aging such as Alzheimer's and Parkinson's diseases and to contribute to aging itself. We have used a screening approach based on RNA interference to isolate genes that contribute to cellular resistance to damage by free radicals. We identified a small interfering RNA that targeted the gene for retinol saturase and led to extremely high resistance to damage by free radicals in cell culture. Retinol saturase is an enzyme that converts vitamin A into a novel retinoid. These results suggest a previously unexpected link between retinoid signaling and cellular resistance to damage by free radicals. It will be interesting to determine how this novel resistance mechanism is manifested in an intact organism and whether it contributes to the diseases of aging.

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## Regulation of the Plasminogen Activation System

L.A. Miles, H. Bai, N. Baik, F.J. Castellino,\* L. Dehmelt, S. Halpain, A. Gutiérrez-Fernández,\*\* R.J. Parmer,\*\*\* V.A. Ploplis\*

\* University of Notre Dame, Notre Dame, Indiana

\*\* Universidad de Oviedo, Oviedo, Spain

\*\*\* University of California, San Diego, California

**A** major role of the plasminogen activation system in neuronal function has been established. Therefore, we have studied the role of the plasminogen activation system in neuritogenesis in hippocampal neurons. When plasminogen was added to primary

cultures of mouse hippocampal neurons, the number of cells with neurites was markedly increased after 24 hours of culture and after 72 hours of culture, the average neurite length was also markedly increased. Similar results were obtained with embryonic rat hippocampal neurons. Furthermore, differential immunostaining of rat hippocampal neurons indicated that the lengths of both axons and dendrites were increased. Conversely, the growth cone areas of the hippocampal neurons were markedly decreased after plasminogen treatment, consistent with the idea that larger growth cones are often stationary and contribute less to neurite outgrowth.

The stimulation of neuritogenesis by plasminogen was abolished in the presence of the plasmin inhibitor aprotinin, suggesting that the activation of plasminogen by endogenous plasminogen activators was required. Therefore, we examined neurite outgrowth in hippocampal neurons of mice deficient in tissue plasminogen activator. Neurite length and the number of hippocampal neurons with neurites did not differ significantly between the experimental mice and the wild-type mice. However, application of amiloride, an inhibitor of urokinase, significantly decreased neurite outgrowth from hippocampal neurons from wild-type mice. Interestingly, the inhibitory effect of amiloride on the length of neurites in hippocampal neurons from the mice lacking the gene for tissue plasminogen activator was significantly greater ( $P < .005$ ) than the effect on neurons from wild-type mice. These results suggest that both neuronal urokinase and neuronal tissue plasminogen activator participate in the outgrowth of hippocampal neurites.

In addition, a direct interaction of plasminogen with the hippocampal neurons appeared to be required, because  $\epsilon$ -aminocaproic acid abolished the enhancement of outgrowth of mouse hippocampal neurons after the addition of plasminogen.  $\epsilon$ -Aminocaproic acid also decreased the number of cells with neurites in hippocampal neurons not treated with plasminogen, suggesting that endogenous neuronal plasminogen is required for optimal neuritogenesis. Accordingly, compared with hippocampal neurons from wild-type mice, neurons from plasminogen-deficient mice had a significant decrease in neurite outgrowth.

These observations suggest that endogenous plasminogen is required for optimal hippocampal neuritogenesis. Furthermore, active plasmin is required. Finally, sufficient plasminogen activation can be accomplished by endogenous urokinase.

Because of the role of endogenous plasminogen in neurite outgrowth, we addressed the effect of a representative neurotrophin, nerve growth factor (NGF), on plasminogen synthesis in model NGF-responsive PC-12 cells. We found that NGF treatment increased plasminogen expression 3-fold and steady-state levels of plasminogen mRNA 6.82-fold. This effect also was observed in cortical neurons. PC-12 cells transfected with a luciferase reporter gene under the control of a 2400-bp fragment of the murine plasminogen promoter had a 5-fold increase in luciferase activity after treatment with NGF. This response depended on Ras/extracellular signal-regulated kinase and phosphatidylinositol-3'-kinase signaling, because treatment with PD98059 together with wortmannin decreased promoter activity in response to NGF to the level of that in untreated cells. Furthermore, cotransfection with a dominant-negative mutant Ha-Ras completely blocked NGF-induced luciferase activity.

In deletional and mutational studies, we identified 2 Sp1 binding sites located between nucleotides -255 and -106 of the plasminogen promoter that were required for the full response of the plasminogen promoter to NGF. In chromatin immunoprecipitation assays, the Sp1 transcription factor bound to the endogenous plasminogen promoter. These results suggest that plasminogen gene expression is upregulated by neurotrophins that may provide a previously unrecognized mechanism for enhancing the effects of neurotrophins via the proteolytic activity of plasmin.

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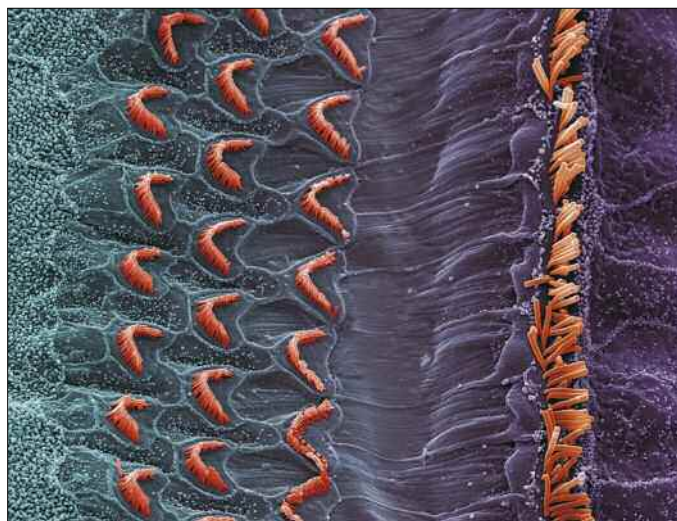
## Auditory Perception and Neuronal Circuit Formation: From Mouse Models to Human Genetic Disease

U. Müller, C. Barros, R. Belvindrah, F. Conti, S. Franco, N. Grillet, S. Harkins-Perry, P. Kazmierczak, I. Martinez-Garay, R. Radakovits, C. Ramos, A. Reynolds, A. Sczaniecka, M. Schwander, S. Webb, W. Xiong

**A** fundamental unresolved question in biology is how the nervous system in humans creates an internal representation of the external world. Sense organs convert signals such as light and sound into electrical impulses that are processed by the nervous system to create a reflection of the surroundings and to elicit appropriate behavioral responses. Selective pressures during evolution have shaped the human genome and optimized sense organs and neuronal circuits for their tasks. Of all the sensory systems in humans, the auditory system is the least well understood at the molecular level. We identify and study genes that control the function of the auditory sense organ of mammals. We also analyze the mechanisms that establish neuronal connections between the auditory sense organs and the cerebral cortex and the formation of cell layers and neuronal circuits within the cortex.

#### AUDITORY PERCEPTION

The ability to perceive sound is critically dependent on mechano-electrical transduction (MET), the conversion of mechanical force into electrical signals. The auditory mechanoreceptor cells in mammals are the hair cells of the cochlea. The architectural features of the cochlea and the properties of the hair cells are essential for encoding time-variant frequency components of sound as spatiotemporal arrays of neural discharge that provide the sense of hearing. The mechanically sensitive organelle of a hair cell is the hair bundle, which consists of dozens of stereocilia that project from the apical cell surface (Fig. 1). MET channels are localized close to the tips of stereocilia. Tip-links, extracellular filaments that connect the tips of neighboring stereocilia and are visible by electron microscopy, are thought to transmit sound-induced tension force onto the MET channel. The molecular identity of most components of the MET complex is still unknown.



**Fig. 1.** Scanning electron microscopy image of the mammalian cochlea. Hair cells are arranged in 3 rows of outer hair cells (to the left) and 1 row of inner hair cells (to the right). Each hair cell contains a bundle of stereocilia (orange) at the apical surface that form the mechanically sensitive organelle of the cell.

To identify genes that control hair cell function, such as the MET channel and the tip-links, we use genetic approaches. Approximately 1 in 1000 children is born deaf, and a large part of the human population experiences age-related hearing loss. Many forms of hearing loss are of genetic origin, and mutations in more than 400 genes cause deafness. Some of the genes have been identified and may encode components of the MET complex in hair cells. Two of the genes linked to deafness encode cadherin 23 and protocadherin 15, members of the cadherin superfamily of cell adhesion molecules. Both genes are expressed in hair cells, and our recent studies indicate that cadherin 23 and protocadherin 15 interact to form tip-link filaments. Thus, we have defined the first components of the MET complex in hair cells at the molecular level. Our findings provide tools for identifying additional components of the MET complex that likely interact with cadherin 23 and protocadherin 15.

In an alternative approach to studies of auditory perception, we carried out a genetic screen in mice. Using *N*-ethyl-*N*-nitrosourea, we introduced point mutations in the germ line of mice. Using phenotypic screens, we identified 19 mouse lines in which the mice inherit hearing defects as recessive traits. We have mapped many of the mutations to chromosomal intervals and have used DNA sequencing to identify mutations in single genes that cause some of the hearing defects.

All of the genes that we have identified so far are expressed in hair cells. Some of the genes encode pro-

teins with known functions, such as myosin motor proteins. Others belong to entirely new gene families that have not been studied previously. Intriguingly, all the genes identified in our screen are also linked to deafness in humans. Therefore, the screen is powerful not only for detecting genes that control the function of hair cells but also for providing animal models for the human disease.

#### NEURONAL CIRCUIT FORMATION

Sensory information is ultimately relayed to specific areas of the CNS such as the auditory and visual cortex. Although the cortex is divided into functional domains, the overall organization of all cortical structures is similar and consists of cell layers that connect to each other to form neuronal circuits. The mechanisms that lead to the establishment of neuronal circuits in the cerebral cortex are mostly unknown. Using genetic tracing studies, we are visualizing neuronal connections that are essential for the processing of auditory signals. In addition, we are defining the genes and mechanisms that lead to the formation of neuronal cell layers. Our studies have already indicated that extracellular matrix receptors of the integrin family have important functions in the formation of cell layers and the control of synaptic function. We are currently using genomic approaches to search for novel genes that specify cortical layers and lead to neuronal circuit formation. We are also participating in a large-scale effort by the National Institutes of Health to generate a panel of mice useful for the perturbation of gene function in defined areas of the CNS, including defined neuronal subtypes in cortical cell layers.

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## Molecular Mechanisms of Thermosensation

A. Patapoutian, M. Bandell, E. Crotty, A. Dhaka, A. Dubin, T. Earley, M. Garret, J. Grandl, L. Macpherson, T. Miyamoto, M. Schmidt, V. Uzzell, B. Xiao

**W**e are interested in the molecular description of the function of sensory neurons. Of the 5 popularly characterized senses—sight, hearing, taste, smell, and touch—touch is among the most varied and least understood. Within this modality is the ability to sense mechanical forces, chemical stimuli, and temperature, and the molecules that mediate this ability have been a long-standing mystery. Temperature sensation in particular has received relatively little attention from biologists and yet is critical for interaction with the environment.

We recently discovered proteins that enable sensory neurons to convey temperature information. These proteins are ion channels activated by specific changes in temperature; thus, they act as the molecular thermometers of the body. Specifically, our results have led to the identification and characterization of 1 novel warm-activated transient-receptor potential (TRP) channel, TRPV3 (33°C threshold), and 2 novel cold-activated TRP channels, TRPM8 (25°C threshold) and TRPA1 (ANKTM1, 17°C threshold). We found that TRPM8 is also the receptor for the compound menthol, providing a molecular explanation of why mint flavors are typically perceived as cooling. Furthermore, we discovered that TRPA1 is activated by cinnamaldehyde, allicin (garlic), and other compounds with a burning sensory quality, consistent with a role of TRPA1 in pain sensation. Together these temperature-activated channels represent a new subfamily of TRP channels that we have dubbed thermoTRPs.

Increasing evidence from *in vivo* studies indicates that thermoTRPs are required for temperature sensation. For example, we recently showed that mice lacking TRPM8 have severe behavioral deficits in response to cold stimuli. In thermotaxis assays of temperature gradient and 2-choice preference tests, TRPM8-deficient mice had strikingly reduced avoidance of cold temperatures. TRPM8-deficient mice also lack behavioral responses to application of cold-inducing icilin and have an attenuated response to acetone, an unpleasant cooling stimulus. Finally, we showed that TRPM8 mediates the analgesic effect of moderate cooling after administration of formalin, a painful stimulus. There-

fore, depending on context, TRPM8 contributes to sensing unpleasant cold stimuli or mediating the effects of cold analgesia.

All organisms have a need to sense the environment. Because some invertebrate species are more amenable to genetic studies than mammals are, we asked whether nonvertebrates also use thermoTRPs. In collaboration with W. Schafer, MRC Laboratory of Molecular Biology, Cambridge, England, we recently showed that the *Caenorhabditis elegans* ortholog of TRPA1 is an ion channel activated by mechanical forces and is required for mechanosensation, suggesting an evolutionary conserved role of TRP channels in somatosensation.

Another key area of research is the mechanism of activation of thermoTRPs. TRPA1 is activated by a variety of noxious stimuli, including cold temperatures, pungent natural compounds, and environmental irritants. How such diverse stimuli activate TRPA1 is unknown. In collaboration with B.F. Cravatt, Department of Cell Biology, and P.G. Schultz, Department of Chemistry, we showed that covalent modification of reactive cysteines within TRPA1 can cause channel activation, rapidly signaling potential tissue damage via the pain pathway. This mechanism of activation is a novel one and raises many important questions about the *in vivo* role of TRPA1 in pain transduction.

Our long-term goal is to synthesize an integrated picture of sensory neuron function. By identifying the proteins that initiate the molecular cascade leading to temperature perception, we have provided the basis for probing the basic foundation of the sense of temperature. We now have the opportunity to extend these insights into important areas of human health, such as pain pathophysiology. For example, TRPA1 is a potential target for treating pain, and we are identifying small-molecule inhibitors of TRPA1 in collaboration with scientists at the Genomics Institute of the Novartis Research Foundation, San Diego, California. Therefore, the approaches we are using will yield insights into the basic biology of the peripheral nervous system and may also have an effect on novel treatments for pain.

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## Functional Proteins in Tumor Metastasis and Angiogenesis

J.P. Quigley, E.I. Deryugina, J.P. Partridge, T. Kupriyanova, V.C. Ardi, E. Conn, C. Li, L.M. Hoffman Hayden

**W**e have established a number of *in vivo* model systems that can recapitulate the major cellular and tissue events that occur during tumor metastasis and angiogenesis. The model systems allow for quantitative measurements, microscopic analysis in real time, biochemical and immunologic probing, and direct molecular and therapeutic intervention.

Recently, use of short interfering RNA molecules directed against specific expressed genes and applied directly in the models have provided insights into the contributory role of the gene products in tumor dissemination and neovascularization. In addition, use of subtractive immunization, which is used to generate unique function-blocking monoclonal antibodies, in combination with immunoproteomics enables us to identify specific antigenic molecules that are functionally active in metastasis and angiogenesis. Finally, the use of activity-based protein profiling, in collaboration with B.F. Cravatt, Department of Cell Biology, enables us to detect, isolate, and identify active proteolytic enzymes that are distinctively and differentially activated during metastasis and angiogenesis.

### METASTASIS

Selected human tumor cells inoculated onto the chorioallantoic membrane of developing chick embryos form primary tumors on the membrane in 4–6 days. A small percentage of the cells in the primary tumor disseminate through the vasculature and arrest and proliferate in secondary organs of the embryo. Measuring a small number of early-arriving metastatic cells (<200) growing and expanding in the secondary organ has always been technically difficult. We use an approach in which unique regions of human DNA, known as *Alu* repeat sequences, are amplified by polymerase chain

reaction from the total DNA extracted from various organs of the tumor-bearing chick embryo. Chicken DNA contains no *Alu* sequences, so any product generated by the polymerase chain reaction indicates that human tumor cells are present in the chick embryo organ and would have arrived there via the known sequential steps in metastasis. We can now detect as few as 25–50 human tumor cells present in the entire chick embryo lung, liver, or brain and can measure the expansion of these metastatic cells by using the real-time polymerase chain reaction.

We are using various screening procedures in this model system to identify molecules that enhance, or conversely inhibit, the appearance of metastatic human tumor cells in organs of chick embryos. The screening procedures include direct inoculation of primary tumor cells that have been transfected with various short interfering RNA constructs to silence specific genes that might contribute to metastatic dissemination. Inoculating monoclonal antibodies directly into the tumor-bearing embryo and monitoring the influence of the antibodies on metastasis are also part of our screening procedures.

We are also monitoring tumor development, expansion, and metastasis in specific immunodeficient mice. With this method, human tumor cells are transplanted to various sites in a mouse and allowed to expand. However, compared with our chick embryo tumor dissemination assay, this method is less quantitative, requires more time (3–6 weeks instead of 3–6 days), and is more difficult to use for inhibitor screening and molecular intervention. We are mainly using the mouse metastasis assay to take advantage of mouse genetics and to confirm the efficacy of various effector molecules and inhibitors that initially are identified in the chick embryo metastasis assay.

### ANGIOGENESIS

One of the more commonly used *in vivo* assays for angiogenesis is the chick embryo chorioallantoic membrane assay. We developed a quantitative variation of this assay that enables us to detect and measure the newly sprouting blood vessels responding to an angiogenic stimulus such as a specific growth factor or a growing tumor. A highly specific metalloproteinase, MMP-13, has been implicated in the tissue remodeling that occurs during the formation of the new blood vessels. We have also implicated another metalloproteinase in angiogenic tissue remodeling, namely MMP-9 (gelatinase B). The proteolytic activity of this enzyme also appears to be necessary for a full angiogenic response.

Interestingly, these 2 critical enzymes are actively imported into the vascular/stromal tissue by distinct inflammatory cells responding to the angiogenic stimulation. Neutrophil-like heterophils rapidly and almost immediately import MMP-9 into the tissue, whereas monocyte/macrophages actively deliver MMP-13 1–2 days later, possibly in response to specific secreted products of the early-arriving heterophils. Thus, normal angiogenesis and tumor angiogenesis are closely linked to an accompanying host inflammatory response that contributes critical functional molecules to the angiogenic process. We are now dissecting out and identifying the specific molecules and cells that link the inflammatory response to angiogenesis and to the progression of malignant neoplasms.

#### INTRAVASATION

We are also investigating intravasation, the entry of primary tumor cells into the host vasculature, including the vasculature that is newly formed during tumor angiogenesis. Intravasation appears to be the least-studied process in the metastatic cascade but likely is a rate-limiting step in tumor dissemination. We recently isolated 2 isogenic variants of a human fibrosarcoma cell line (HT1080) that differ 100-fold in their ability to enter the vasculature *in vivo* and in their ability to metastasize. We are using array technology, proteomic approaches, and intravital microscopy in the cellular and molecular analysis of these 2 variants. The results should indicate specific molecules that are functionally important in interactions between tumor cells and the vasculature and contribute to intravasation. Using activity-based protein profiling, we recently found that the proteolytic enzyme urokinase is differentially activated during intravasation and catalytically contributes to the enhanced entry of tumor cells into the vasculature.

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## Regulation of Gene Expression in Response to Nutrients

E. Saez, N. Mitro, J. Pawlak, C. Godio

**W**e are interested in how nutrients are sensed and in diet-modulated signaling pathways that alter gene expression to control energy balance in mammals. Proper regulation of nutrient-sensitive metabolic pathways is critical to ensure health and survival of the organism. Defective control of metabolic pathways is associated with multiple serious disorders, including cancer, obesity, and diabetes. By understanding how diet influences gene expression, we aim to uncover novel therapeutic targets for the treatment of metabolic diseases such as obesity and diabetes.

Nuclear receptors are ligand-activated transcription factors that modulate gene expression in response to endocrine and environmental signals. Members of the family that work as heterodimers with the retinoid X receptor are sensors of various dietary components, including lipids, fatty acids, retinoids, vitamins, cholesterol, bile acids, and xenobiotics. The liver X receptor (LXR) is a nuclear receptor that is activated by oxidized forms of cholesterol (oxysterols). It acts as a sensor of excessive intracellular accumulation of pathogenic forms of cholesterol and activates a program of gene expression to promote removal of harmful cholesterol. In the liver, LXRs also control triglyceride production by regulating expression of the enzymes responsible for fatty acid synthesis. In addition, LXR $\alpha$  and LXR $\beta$  also modulate expression of key genes in glucose metabolism.

As a follow up to this research, we recently discovered that glucose can bind LXRs and activate LXR target genes *in vivo*. This novel carbohydrate signaling pathway appears to determine the fate of glucose in the liver; excess glucose is sensed by the same transcription factor responsible for control of fatty acid synthesis. LXRs appear to be the molecular switch responsible for transforming surplus energy into triglycerides to be stored in fat tissue for times of deprivation. LXRs also behave as a glucose sensor in the intestine, where activation of the receptors by glucose may affect the metabolism of cholesterol and fatty acids.

Spread of the Western diet and a sedentary lifestyle have led to a spectacular increase in the prevalence of obesity, type 2 diabetes, and cardiovascular disease. The risk for atherosclerosis is much greater in people with diabetes than in healthy individuals. The molecu-

lar details of how hyperglycemia facilitates atherogenesis remain poorly understood. Because the other natural ligands of LXRs are oxysterols, it is tempting to speculate that as dual cholesterol-glucose sensors, LXRs may be a molecular connection between diabetes and atherosclerosis.

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## Mechanisms Governing Clathrin-Mediated Endocytosis

S.L. Schmid, S. Archarya, J. Chappie, M. Leonard, V. Lukiyanchuk, M. Mettlen, T. Pucadyil, R. Ramachandran, M.C. Surka, D. Yarar

Clathrin-mediated endocytosis is essential for the efficient uptake of nutrients and other macromolecules into cells and for the regulation of signaling by cell-surface receptors. The process occurs at clathrin-coated pits, which concentrate receptor-ligand complexes, deform the membrane, invaginate, and eventually pinch off, forming clathrin-coated vesicles. Formation of these vesicles requires clathrin and adaptors, the major coat proteins, and numerous accessory factors, including the atypical GTPase family member dynamin, which we suggest plays a dual role as a regulator at early stages and in membrane fission at late stages of formation of clathrin-coated vesicles. In a multidisciplinary approach, we are using biochemistry, cell biology, live-cell microscopy, biophysics, and structure biology to probe the underlying mechanisms that govern endocytosis.

Multiple endocytic pathways, including clathrin-mediated endocytosis, also require actin-dependent remodeling of the plasma membrane. However, neither the factors linking these processes nor the factors'

mechanisms of action are understood. We found that the dynamin-binding partner sorting nexin 9 (SNX9) may be a molecular link between actin assembly and endocytosis. In addition to its role in clathrin-mediated endocytosis, SNX9 is also critical for other clathrin-independent, actin-dependent fluid-phase endocytic pathways. SNX9 directly associates with and activates neural Wiskott-Aldrich syndrome protein, a regulator of actin assembly, which stimulates the actin-related protein 2/3-dependent assembly of highly branched actin filaments *in vitro*. This assembly is greatly enhanced in the presence of liposomes containing phosphatidylinositol 4,5-bisphosphate that mimic the composition of the plasma membrane. Our findings suggest a novel mechanism for the spatial and temporal regulation of neural Wiskott-Aldrich syndrome protein-dependent actin assembly and implicate SNX9 in directly coupling actin dynamics to membrane remodeling during multiple modes of endocytosis.

Previously, we showed that dynamin self-assembles into rings and helical stacks of rings that form "collars" around the necks of deeply invaginated coated pits. We proposed that assembly-stimulated GTP hydrolysis by dynamin triggers a conformational change in the assembled collar to constrict the necks and facilitate membrane fission. We have begun to take a combined biophysical and structural approach to define the mechanochemical properties of assembled dynamin. Thus, we have developed real-time fluorescence-based assays to detect dynamin-liposome interactions, dynamin self-assembly, and nucleotide-dependent conformational changes of assembled dynamin on liposomes. In collaboration with R.A. Milligan, Department of Cell Biology, we are using electron cryomicroscopy and helical reconstruction techniques to determine the structure of assembled dynamin on liposomes in distinct guanine nucleotide-bound states. We are also developing new assays with supported lipid bilayers in an effort to reconstitute formation of coated vesicles and membrane fission in a biochemically defined system. To test hypotheses that emerge from these studies on the structural requirements for dynamin function in endocytosis, we have developed cells in which the gene for dynamin-2 can be inactivated under certain conditions. We will reconstitute the cells with various dynamin mutants for *in vivo* structure-function analyses.

Finally, we have used total internal reflection fluorescence microscopy to follow the dynamics of the assembly of coated pits and the formation of coated

vesicles by tracking fluorescently labeled clathrin and adaptors in live cells. Our collaborator, G. Danuser, Department of Cell Biology, has developed sophisticated tracking software and statistic analysis methods to detect kinetically distinct subpopulations of coated pits at the cell surface. Together, to functionally define these subpopulations, we are examining the effects of cargo concentration and dynamin on the kinetic behavior and distribution of coated pits.

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## Molecular Biology of Olfaction

L. Stowers, P. Chameró, K. Flanagan, D. Logan, T. Marton, F. Papes, C. Ramos

Every breath samples the environment for olfactory chemical information, determining the quality of food, warning of danger, and confirming safety. The neurons that mediate olfaction are of 2 types: those that mediate an evocative perception that varies with an individual's experience and those that regulate stereotyped innate social behaviors such as aggression and mating in response to pheromones. The biochemical nature of the pheromone code that induces innate behavior in mammals is not known, and the sensory system responsible for the detection of pheromones is a matter of controversy. We expect that elucidating the stimulating ligands and responsive neurons will enable us to activate, study, and identify the mechanisms underlying neural information coding of defined behaviors.

We recently isolated novel mouse pheromones that mediate aggressive behavior and determined the identity of the aggression-promoting chemosensory neurons. We have taken a unique approach to decode olfaction; we are working forward from the behavioral activity to identify novel genetically encoded pheromones. These pheromone proteins reproducibly activate a subset of

accessory olfactory neurons, enabling the molecular identification of those neurons that mediate the aggression-promoting behavior. This step is a fundamental one for understanding intraspecific communication in mammals and for characterizing the neuronal circuit that regulates behavior.

The response of an individual to pheromones varies with the individual's age, sex, and dominance. To investigate the molecular mechanisms that generate this neuronal plasticity, we are elucidating the ligands and responsive neurons that mediate female-, infant-, and dominant male-specific behaviors, as well as predator responsive circuits that are not thought to be plastic. We expect to determine the extent to which the plasticity is occurring in the sensory neurons or more centrally in the amygdala or hypothalamus of the brain. Furthermore, we will have the molecular tools to determine both the logic underlying each behavior and the more general mechanisms of neuronal regulation and activation.

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## Nicotinic Acetylcholine Receptor

N. Unwin

The nicotinic acetylcholine receptor is a member of a superfamily of transmitter-gated ion channels, which includes the receptors for serotonin,  $\gamma$ -aminobutyric acids A and C, and glycine. It has a cation-selective pore, delineated by a ring of 5 similar subunits, that opens upon binding of acetylcholine to the 2 ligand-binding ( $\alpha$ ) subunits. My colleagues and I are interested determining how this channel works. For our studies, we use electron microscopy to analyze the structure of the channel trapped in different physiologic states. We use postsynaptic membranes isolated from the (muscle-derived) electric organ of the *Torpedo* ray, which form tubular crystals of acetylcholine receptors.

Recently, we derived a refined atomic model of the acetylcholine receptor in the closed-channel form. We found that the individual subunits in the N-terminal ligand-binding domain are organized around 2 sets of  $\beta$ -sheets packed in a curled  $\beta$ -sandwich, as in the related soluble pentameric acetylcholine-binding protein. Each of the subunits in the membrane-spanning domain is made from 4  $\alpha$ -helical segments. The heli-

cal segments arrange symmetrically, forming an inner ring of helices that shape a water-filled pore and an outer shell of helices that coil around each other and shield the inner ring from the lipids. In the closed channel, the helices in the inner ring come together near the middle of the membrane and make a constricting hydrophobic girdle. This girdle, which is about 50 Å from the acetylcholine-binding sites, constitutes an energetic barrier to ion permeation and functions as the gate of the channel.

These details, together with those obtained earlier from studies of the receptor trapped in the open-channel form, have enabled us to understand in outline the conformational change triggered by acetylcholine that opens the gate of the channel. Improvements in resolution of the structure, in both the closed- and the open-channel forms, are now being attempted so that the gating mechanism can be described in atomic detail. These studies are yielding crucial insight into the nature of a number of neuromuscular disorders, including several well-characterized congenital myasthenic syndromes. They are also providing important 3-dimensional information about the binding sites for drugs that affect the brain by modulating the function the related  $\gamma$ -aminobutyric acid, serotonin, glycine, and neuronal acetylcholine receptors.

## Advancing Applications in Mass Spectrometry–Based Proteomics

J.R. Yates III, E. Chen, D. Cociorva, J. Coppinger, C. Delahunty, M.Q. Dong, X. Han, J.R. Johnson, L. Liao, B.W. Lu, D. McClatchy, A. Nakorchevskiy, S.K. Park, H. Prieto, C.I. Ruse, M. Wang, C. Wong, T. Xu

**M**ass spectrometry has emerged as a powerful technique for cellular proteomics, complementing traditional gene-by-gene approaches with a comprehensive description of the molecular factors that contribute to a biologically relevant system. We remain at the forefront of this field, developing new strategies to address more sophisticated scientific questions through proteomics, such as how to measure global changes in protein abundance and how to characterize complex posttranslational modifications.

### QUANTITATIVE PROTEOMIC ANALYSIS OF INSULIN SIGNALING

To understand the downstream targets of insulin/insulin-like growth factor 1 (IGF-1) signaling, we used

quantitative mass spectrometry to identify proteins whose levels change upon perturbation of insulin signaling in the worm *Caenorhabditis elegans*. We found 104 such proteins encoded by 91 genes, including previously known insulin/IGF-1 signaling targets. The accuracy of our results was independently confirmed by using Western blotting of 9 proteins. Among the proteins upregulated in *daf-2* (insulin/IGF-1 receptor) mutants, those that functioned in gluconeogenesis, amino acid biosynthesis, and the removal of reactive oxygen species were overrepresented. Among the proteins downregulated in *daf-2* mutants, those involved in translation and lipid transport were overrepresented.

Most of the targets identified in this study were not found previously by mRNA profiling. Genetics analysis of some of the novel targets confirmed that the targets participate in insulin/IGF-1–regulated processes in *C. elegans*, such as formation of dauer larvae and aging. We found that one target, the protein phosphatase calcineurin, positively regulates insulin signaling. The calcineurin inhibitors cyclosporin A and FK506 increase the risk of new-onset diabetes mellitus after transplantation in humans, suggesting that calcineurin might also regulate insulin signaling in humans. Our results illustrate the effectiveness of quantitative mass spectrometry in profiling changes in protein abundance and providing new insights to the insulin signaling network.

### QUANTITATIVE PROTEOMIC ANALYSIS OF ORGAN-SPECIFIC BREAST CANCER METASTASIS

In the past, the invasive properties of metastatic cancer cells and the ability of the cells to attract new blood vessels received much attention. However, intrinsic mechanisms that allow metastatic tumor cells to survive and proliferate in preferred target organs are poorly understood. For a better understanding of the nature of breast cancer cells that cause distal metastases, we analyzed protein expression profiles of tumor cells from primary, bone, and brain metastatic breast cancer cells to gain insight into the specific cellular properties that promote tumor cell survival in the unique secondary microenvironment.

Brain metastases are the most feared complication in breast cancer. Nearly 20% of patients with advanced breast cancer eventually have brain lesions, making breast tumors the main source of metastatic brain disease in women. The incidence of brain metastases is increasing as patients respond to improved cancer therapy and live longer, but no current regimen significantly affects breast cancer brain metastases. In order to

develop effective regimens that prevent and control this stage of the disease, a better understanding of the nature and functionality of breast cancer cells that cause brain metastases is needed.

To define determinants of breast cancer cell growth in the brain, we used multidimensional protein identification technology to analyze the protein expression profiles of the parental cell line and its brain- or bone-homing variants. We found more than 300 proteins that are uniquely regulated in the brain metastatic cells. Most of these proteins are involved in cellular metabolism and the response to cell stress.

Our proteomic results, transcriptional validation, and cell function analyses *in vitro* indicate that brain metastatic cells use enhanced mitochondrial respiratory pathways for energy production and antioxidant defense mechanisms. Furthermore, our findings suggest that functional adaptation of metastatic breast cancer cells to the brain as a target tissue is associated with specific molecular changes, which likely provide a growth advantage within that unique microenvironment. Last, our results indicate the importance of understanding the metabolic state of metastatic cells and the value of global proteome analyses.

#### STABLE ISOTOPIC LABELING IN MAMMALS

The introduction of stable isotope labels into mammals used as models for human physiology and disease creates an opportunity to study a wide range of diseases that affect tissues and organs. To pursue global quantification of complex mammalian tissues with mass spectrometry, we developed a technique, stable isotopic labeling of mammals, to metabolically label a rat by feeding it a  $^{15}\text{N}$ -enriched protein diet. We showed that the labeled rat was healthy and phenotypically identical to an unlabeled rat. Using the proteins from the liver of the  $^{15}\text{N}$ -labeled rat as internal standards, we quantified global changes in the liver induced by a sublethal dose of cyclohexamide. In addition, we verified the usefulness of our new protocol by quantifying developmental changes in the brain. Besides finding excellent correlation with the existing literature on brain development, we identified potential novel regulators of brain development. This labeling method will allow quantitative mass spectrometry analysis of animal models of neurologic disorders or other types of diseases.

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## Membrane Proteins and Viruses Visualized by Electron Cryomicroscopy and Image Analysis

M. Yeager, R. Abagyan,\* B.D. Adair, G.A. Altenberg,\*\* K. Altieri, M.A. Arnaout,\*\*\* K.A. Baker, A.R. Bellamy,\*\*\*\* N. Ben-Tal,\*\*\*\*\* M.J. Buchmeier,\* A. Cheng, F.V. Chisari,\* K. Coombs,† M.J. Daniels, K.A. Dryden, B. Ganser, H.B. Greenberg,†† J. Harless, Y. Hua, J.E. Johnson,\* M. Matho, S. Matsui,†† L.H. Philipson,††† A. Rein,†††† A. Schneemann,\* J.A. Tainer,\* J.A. Taylor,\*\*\*\* V.M. Unger†††††

\* Scripps Research

\*\* Texas Tech University Health Sciences Center, Lubbock, Texas

\*\*\*\* Harvard Medical School, Boston, Massachusetts

\*\*\*\* University of Auckland, Auckland, New Zealand

\*\*\*\*\* Tel-Aviv University, Tel-Aviv, Israel

† University of Manitoba, Winnipeg, Manitoba

†† Stanford University, Stanford, California

††† University of Chicago, Chicago, Illinois

†††† National Cancer Institute, Frederick, Maryland

††††† Yale University, New Haven, Connecticut

The ultimate goal of our studies is to gain a deeper understanding of the molecular basis of important human diseases, such as sudden death, heart attacks, and HIV infection, that cause substantial mortality and suffering. The structural details revealed by our research may provide clues for the design of more effective and safer medicines.

At the basic science level, we are intrigued by questions at the interface between cell biology and structural biology: How do membrane proteins fold? How do mem-

brane channels open and close? How are signals transmitted across a cellular membrane when an extracellular ligand binds to a membrane receptor? How do viruses attach to and enter host cells, replicate, and assemble infectious particles? To explore such problems, we use high-resolution electron cryomicroscopy and computer image processing. With this approach, we can examine the molecular architecture of supramolecular assemblies such as membrane proteins and viruses.

In electron cryomicroscopy, biological specimens are quick frozen in a physiologic state to preserve their native structure and functional properties. A special advantage of this method is that we can capture dynamic states of functioning macromolecular assemblies, such as open and closed states of membrane channels and viruses actively transcribing RNA. Three-dimensional density maps are obtained by digital image processing of the high-resolution electron micrographs. The rich detail in the density maps exemplifies the power of this approach to reveal the structural organization of complex biological systems that can be related to the functional properties of such assemblies.

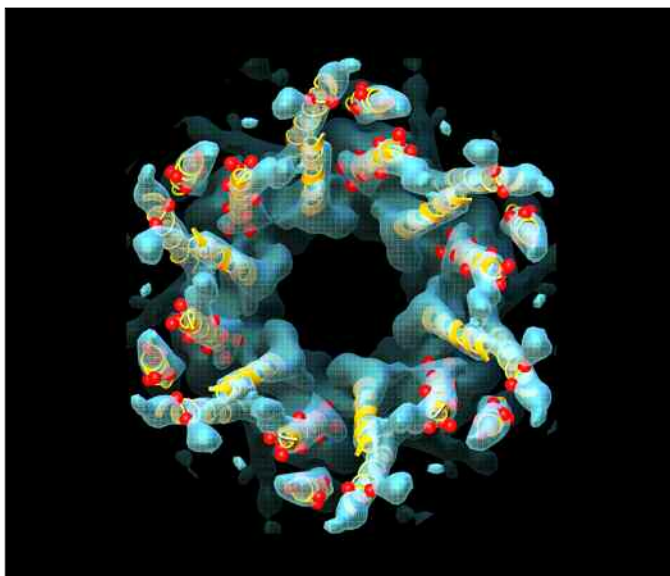
Ongoing research projects include the structure analysis of (1) membrane proteins involved in cell-to-cell communication (gap junctions), water transport (aquaporins), ion transport (potassium channels), transmembrane signaling (integrins), and viral recognition (rotavirus NSP4); (2) viruses responsible for significant human diseases (retroviruses, hepatitis B [HBV], rotavirus, astrovirus); and (3) viruses used as model systems to understand mechanisms of pathogenesis (arenaviruses, reoviruses, nodaviruses, tetraviruses, and sobemoviruses). The following sections summarize selected projects that exemplify the themes of our research program.

#### GAP JUNCTION MEMBRANE CHANNELS

Gap junction channels connect the cytoplasm of adjacent cells by means of an intercellular conduit formed by the end-to-end docking of 2 hexameric hemichannels called connexons. Gap junctions play an essential functional role by mediating metabolic and electrical communication within tissues. For instance, in the heart, gap junction channels organize the pattern of current flow to allow a coordinated contraction of the muscle.

We expressed a recombinant cardiac gap junction protein, termed connexin 43, and produced 2-dimensional crystals suitable for electron cryocrystallography. Our previous findings indicated that each hexameric connexon is formed by 24 closely packed  $\alpha$ -helices. We extended this analysis to 5.7-Å in-plane and 19.8-Å

vertical resolution, a step that enabled us to identify the positions and tilt angles for the 24  $\alpha$ -helices within each hemichannel (Fig. 1). The 4 hydrophobic segments



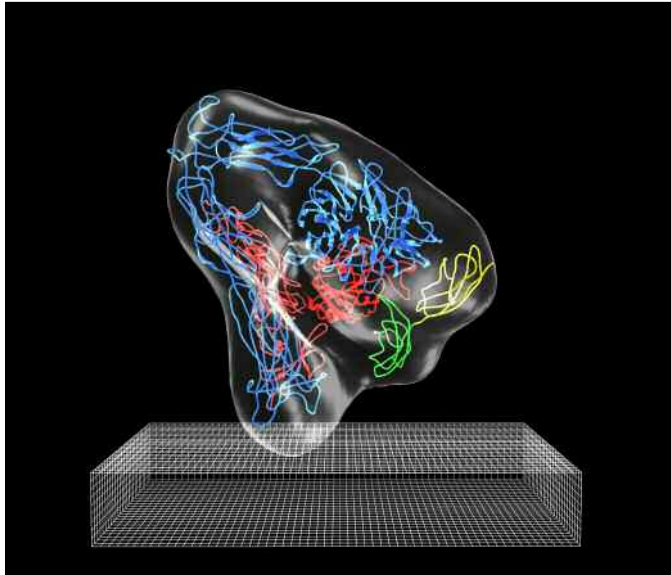
**Fig. 1.** Intercellular gap junction channels have a diameter of about 65 Å and are formed by the end-to-end docking of 2 hemichannels, each of which is composed of a hexamer of connexin subunits. A  $C\alpha$  model (ribbons) for the membrane-spanning  $\alpha$ -helices of the hemichannels was derived by combining the information from a computational analysis of connexin sequences, the results of more than a decade of biochemical studies, and the constraints provided by a 3-dimensional map derived by electron cryocrystallography. Although individually none of these approaches provided high-resolution information, their sum yielded an atomic model that predicts how connexin mutations (spheres), which result in diseases such as nonsyndromic deafness and Charcot-Marie-Tooth disease, may interfere with formation of functional channels by disrupting helix-helix packing.

in connexin sequences were assigned to the  $\alpha$ -helices in the map on the basis of biochemical and phylogenetic data. Evolutionary conservation and an analysis of compensatory mutations in connexin evolution were used to identify the packing interfaces between the helices. The final model, which specifies the coordinates of  $C\alpha$  atoms in the transmembrane domain, provides a structural basis for understanding the different physiologic effects of almost 30 mutations and polymorphisms in terms of structural deformations at the interfaces between helices, revealing an intimate connection between molecular structure and disease.

#### INTEGRINS

Integrins are a large family of heterodimeric transmembrane receptor proteins that modulate important biological processes such as development, cell adhesion, angiogenesis, wound healing, and neoplastic transformation. The ectodomain of the integrin  $\alpha_v\beta_3$  crystallizes in a bent conformation, which is considered to be inactive

(i.e., unable to bind physiologic ligands in solution) unless it is fully extended by activating stimuli. To assess whether the bent integrin can bind physiologic ligands, we collaborated with M.A. Arnaout, Harvard Medical School, Boston, Massachusetts, to generate a stable, soluble complex of the manganese-bound  $\alpha_v\beta_3$  ectodomain with a fragment of fibronectin containing type III domains 7–10 and the EDB domain. Electron microscopy and single-particle image analysis were used to determine the 3-dimensional structure of this complex (Fig. 2).



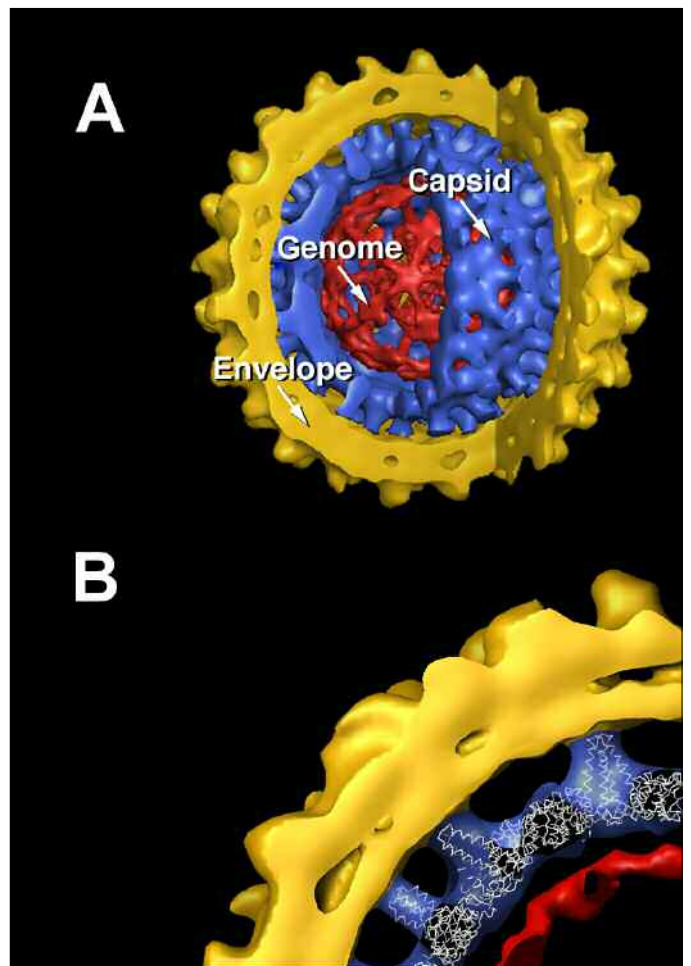
**Fig. 2.** The 3-dimensional density map (gray-scale transparency) of the integrin  $\alpha_v\beta_3$  in a complex with fibronectin was determined by using electron microscopy and image analysis. The x-ray structures of the  $\alpha_v$  and  $\beta_3$  proteins have been docked into the electron microscopy density envelope. Additional density (lower right) can accommodate fibronectin domain 10 adjacent to the ligand-binding site as well as domain 9 at the synergy site. The complex is shown adjacent to the white box, which represents the 30-Å-thick hydrophobic part of the cellular membrane across which signals are transmitted.

Most  $\alpha_v\beta_3$  particles, whether ligand-free or bound to fibronectin, had compact, triangular shapes. A difference map comparing ligand-free and fibronectin-bound integrin revealed density that could accommodate the fibronectin type III domain 10 containing arginine–glycine–aspartic acid in proximity to the ligand-binding site of  $\beta_3$ , with domain 9 just adjacent to the synergy site, binding region of  $\alpha_v$ .

This study suggests that the ectodomain of  $\alpha_v\beta_3$  has a bent conformation that can stably bind a physiologic ligand in solution. These results are relevant for understanding how binding of ligands to the extracellular domain leads to conformational changes that transmit signals across the plasma membranes of cells, culminating in changes in gene transcription in the nucleus.

## HEPATITIS B VIRUS

HBV infects more than 350 million people, of which 1 million will die every year. The infectious virion is an enveloped capsid containing the viral polymerase and the double-stranded DNA genome. The structure of the capsid assembled in vitro from expressed core protein has been studied intensively. However, little is known about the structure and assembly of native capsids present in infected cells, and even less is known about the structure of mature virions. We used electron cryomicroscopy and image analysis to examine HBV virions (also called Dane particles) isolated from the serum of a patient with HBV infection and capsids positive and negative for HBV DNA isolated from the livers of transgenic mice (Fig. 3).



**Fig. 3.** A model of the HBV virion (diameter about 450 Å) based on electron cryomicroscopy and image analysis. A, The double-stranded DNA genome is encapsidated by an icosahedral capsid shell composed of 120 spikes. The surface is studded with glycoproteins spaced about 60 Å apart that bind to membrane receptors on liver cells. B, In the close-up view, the x-ray crystal structure of a recombinant capsid has been docked into the electron cryomicroscopy density map of the virion capsid. The core spikes are in close apposition but do not penetrate the envelope.

Both types of capsids assembled as icosahedral particles indistinguishable from previous image reconstructions of capsids. Likewise, the virions contained capsids with either  $T = 3$  or  $T = 4$  icosahedral symmetry. Projections extending from the lipid envelope were attributed to surface glycoproteins. The packing of the projections was unexpectedly nonicosahedral but conformed to an ordered lattice. These structural features distinguish HBV from other enveloped viruses.

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