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Joshua Zimmerberg: The Biophysics of Protein-Lipid Interactions in Influenza and Coronavirus, Malaria, and Muscular Dystrophy
Colophon
Board of Scientific Counselors

* nominee

Elizabeth Bonney, MD, MPH, Chair  
7/1/17 – 6/30/22  
Immunobiology, Molecular Biology, Obstetrics, and Gynecology  
Professor and Head, Division of Reproductive Sciences, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Vermont College of Medicine

Kate G. Ackerman, MD  
7/1/16 – 6/30/21  
Developmental Biology, Genetics, and Pediatrics  
Associate Professor, Departments of Pediatrics and Biomedical Genetics, University of Rochester Medical Center, School of Medicine and Dentistry

Hugo J. Bellen, DVM, PhD*  
7/1/19 – 6/30/24  
Genetics, Neuroscience, Model Organisms  
Investigator, Howard Hughes Medical Institute; Professor, Departments of Molecular and Human Genetics and Neuroscience, Baylor College of Medicine

Serdar E. Bulun, MD  
7/1/16 – 6/30/21  
Obstetrics and Gynecology  
John J. Sciarra Professor and Chair, Department of Obstetrics and Gynecology, Northwestern University Feinberg School of Medicine; Chief, Division of Obstetrics and Gynecology-Reproductive Biology Research, Prentice Women's Hospital

Nancy Carrasco, MD*  
7/1/19 – 6/30/24  
Molecular Medicine, Pharmacology, and Physiology  
Professor and Chair, Department of Molecular Physiology and Biophysics, Vanderbilt School of Medicine

William T. Dauer, MD  
7/1/18 – 6/30/23  
Neurodevelopment and Behavior, Rare Diseases and Genetics, Neurobiology  
Director, Peter O'Donnell Jr. Brain Institute; Professor of Neurology and Neurotherapeutics, and Neuroscience, Lois C.A. and Darwin E. Smith Distinguished Chair in Neurological Mobility Research, UT Southwestern Medical Center

P. Ellen Grant, MD*  
7/1/19 – 6/30/24  
Newborn Medicine, Radiology  
Founding Director, Fetal-Neonatal Neuroimaging and Developmental Science Center; Professor of Radiology and Pediatrics, Harvard Medical School; Endowed Chair in Neonatology, Boston Children's Hospital
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<td>President and Chief Executive Officer of Keystone Symposia; Professor, Department of Molecular and Cellular Biology, Baylor College of Medicine</td>
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<td>Ursula Kaiser, MD*</td>
<td>Chief, Division of Endocrinology, Diabetes, and Hypertension; Co-Director, Brigham Research Institute, Brigham and Women's Hospital; Professor of Medicine, Harvard Medical School</td>
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<td>Errol Norwitz, MD, PhD, MBA*</td>
<td>President and CEO, Newton-Wellesley Hospital</td>
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<td>Linda Overstreet-Wadiche, PhD*</td>
<td>Professor, Department of Neurobiology, University of Alabama at Birmingham</td>
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<td>Eric Vilain, MD, PhD</td>
<td>A. James Clark Distinguished Professor of Molecular Genetics; Director, Center for Genetic Medicine Research, Children's Research Institute, Children's National Medical Center</td>
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<td>Martha M. Werler, DSc</td>
<td>Professor and Chair, Department of Epidemiology, School of Public Health, Boston University</td>
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Message from the Scientific Director

The Division of Intramural Research (DIR) of the Eunice Kennedy Shriver National Institute of Child Health and Human Development, like other institutions across the world, was impacted by the SARS-CoV-2 virus and COVID-19 pandemic. In March 2020, labs had to put nearly all of their experiments on hold. Our scientists began returning to the lab during the summer, but as of this writing are still only working at about 40% capacity. Despite this, our laboratories have remained scientifically engaged and productive.

Our 2020 annual report is now available to you electronically, either on the web and on your cell phones or tablets, at: http://annualreport.nichd.nih.gov

We invite you to look through the report site, to review our medical and scientific discoveries of the past year, to see what work a colleague may currently be engaged in, or to identify a laboratory where you may wish to collaborate or refer a student. For potential postdoctoral fellows, graduate students, and clinical fellows, the report is fully searchable. It offers you an introduction to the array of research endeavors in NICHD’s DIR.

NICHD intramural investigators comprise a broad array of basic, translational, and clinical researchers. Our work is reflected in our mission statement:

“To plan and conduct the Institute’s laboratory and clinical research programs to seek fundamental knowledge about the nature and behavior of living systems through basic, clinical, and population-based research and determine how to apply such knowledge to illuminate developmental origins of health and disease and help ensure that women and men have good reproductive health, that children are born healthy, and that people develop to live healthy and productive lives.”

We use a range of model systems in the areas of developmental biology, molecular and cellular biology, neurosciences, structural biology, imaging, and biophysics. Investigators take advantage of our resources in a large zebrafish core facility and working with a number of other animal models, from fruit flies to rats and mice, and supported by a wide array of core services, including bioinformatics, imaging, and molecular genomics. Each investigator participates in at least one, and typically more, affinity groups. These affinity groups are team-based and future-oriented—to build on thematic interests while responding to rapidly shifting scientific priorities as new knowledge is uncovered.

I invite you to read through the selection of our Clinical Research Protocols listed in this report and to consider how we may collaborate, through the NIH U01 grant mechanism at the NIH Clinical Research Center. The support of this program can lead to our next new success in therapeutics, the next miracle drug, if we combine expertise, take advantage of our NIH infrastructure and our patient population, whether on rare disorders or the most persistent problems affecting human health. You can learn more about the U01 opportunities at https://ocr.od.nih.gov/new_u01/new_u01.html.
The DIR researchers whose names appear in this publication are committed to training the next generation of scientists and physician scientists; they include tenure-track investigators who have recently joined us and accomplished investigators who continue to forge new scientific paths. Link to their reports on the web to learn about their work in 2020. I also invite you to reach out to me with your ideas and proposals for collaborative initiatives we may undertake together, at dassom@mail.nih.gov.

Our drive and purpose, on behalf of the American public and the international community, is to strive to uncover the fundamental principles that underpin reproduction and development, as well as to apply these findings to improve human reproductive health and the healthy growth and maturation of children and adolescents. This is our privilege and responsibility to our chosen professions.

Sincerely yours,

Mary C. Dasso, PhD
Acting Scientific Director, NICHD, NIH
December 2020
Office of the Scientific Director

The Office of the Scientific Director (OSD) is responsible for programmatic oversight, review, and administration of the Division of Intramural Research (DIR), NICHD. The Office has several key functions including:

- Serving our staff, investigators, administrators, trainees, and contractors to meet their needs and ensure that we are all working together to serve the mission of NICHD.
- Planning, coordinating, and directing the basic and clinical scientific research programs conducted within the DIR, all with the advice of the Board of Scientific Counselors (BSC; see below) and in consultation with the Institute's Director and other offices.
- Providing oversight of scientific organization, setting of overarching research priorities, and allocation of resources.
- Coordinating the recruitment and review of principal investigators and other key staff.
- Providing oversight of scientific mentoring and training activities, and, as a high priority, ensuring that we provide state-of-the-art training in basic, translational, and clinical research for the next generation of scientific and clinical leaders.
- Encouraging the implementation of new technologies and the application of new techniques and treatments through clinical trials, and promoting research collaboration.
- Developing and maintaining the necessary scientific capability in the DIR to foster and guide an effective research and clinical program.
- Ensuring compliance with safety, ethics, and other legal and policy requirements.
- Providing clearance of manuscripts and other publications and ensuring the deposition of data and published manuscripts in appropriate public databases (e.g., PubMedCentral, Clinicaltrials.gov).
- Serving in an advisory role to the Institute Director and other senior leadership of the Institute and other institutes, offices, and centers across NIH.
- Actively participating in the governance of the NIH Intramural Research Program (IRP).
- Contributing to the development and support of trans-NIH initiatives.
- Working closely with other intramural research programs across NIH to support scientific and other efforts across institutes.

To maintain the highest quality of research, principal investigators and other key staff of the DIR are evaluated by the BSC, which meets biannually on the first Friday of December and in June of each year. The
BSC reviews site-visit reports, evaluations, and all other activities of the OSD. Each NICHD investigator is subject to a review at least once every four years, according to NIH policy (NIH Sourcebook Process for Reviewing NIH Intramural Science, [https://oir.nih.gov/sourcebook/processes-reviewing-nih-intramural-science](https://oir.nih.gov/sourcebook/processes-reviewing-nih-intramural-science)). To ensure the most effective use of public dollars toward high-quality, high-impact research, the Board is made up of accomplished senior extramural researchers. Membership of the BSC is listed at [https://annualreport.nichd.nih.gov/bsc.html](https://annualreport.nichd.nih.gov/bsc.html).

Annually, the Scientific Director reports on the activities of the OSD, the reviews of our investigators and their accomplishments, and on all BSC recommendations to NICHD’s National Advisory Child Health and Human Development (NACHHD) Council ([https://www.nichd.nih.gov/About/Advisory/Council](https://www.nichd.nih.gov/About/Advisory/Council)).
Office of Education

The goals and objectives of the Office of Education can be summarized as follows: to meet the training needs of intramural scientists, fellows, and students at all levels, the Office of Education recruits and develops academic support programs, contributes to mentoring, evaluation, and career guidance, and creates new training initiatives. Our professional development workshops and activities typically include public speaking workshops, job interviewing, writing and editorial services for professional school and job applications, grantsmanship workshops, academic and non-academic career presentations, one-on-one counseling, teaching opportunities, and lab management programs. Additional areas of involvement include programming for career exploration, networking among fellows and alumni, grantsmanship, and the enhancement of fellows’ competitiveness for research awards and future career opportunities, as well as support of tenure-track investigators.

Notable accomplishments of the past year
The Office of Education organizes numerous workshops, programs, and individualized opportunities for a population averaging 250 trainees, including: postdoctoral, visiting, and research fellows; clinical fellows and medical students; graduate students; and postbaccalaureate fellows, as well as summer trainees.

We continued our Graduate Student Talks initiative, established in 2014, which provides the NICHD graduate students with experience in presenting their thesis research to a non-specialist scientific audience.

An online Annual Progress Review for fellows, developed by the office and launched in 2016, tracks scientific and career development and progress. As part of the assessments of investigators’ mentoring, the reports by fellows are analyzed and provided to the site visits of the Board of Scientific Counselors.

In November 2020, the Division of Intramural Research (DIR) gave its 13th Mentor of the Year Awards to Dr. Brant Weinstein, Section on Vertebrate Organogenesis, DIR, and Dr. Leah Lipsky, Social and Behavioral Sciences Branch, Division of Intramural Population Health Research (DIPHR), in the investigator category, and to Dr. Hyun Min Jung, Section on Vertebrate Organogenesis, DIR, as fellow.

Our TmT (Three-minute Talks) competition was postponed until 2021 because of the COVID-19 pandemic.
LEADERSHIP AND MANAGEMENT
• Project management for scientists
• Organizational dynamics and supervisory skills
• Setting expectations and goals
• Improving lab communication

GRANTS
• Grant writing/review workshops
• Mock study sections
• Individualized coaching for the NIH application process
• NICHD Fellows Intramural Grants Supplement (FIGS) Award

RESEARCH ETHICS
• An established training plan for all fellows
• 8-hour requirement, within the initial two years of training
• Completed by the fellow or facilitated by the mentor or Office of Education

RESEARCH AWARDS
• Annual Fellows Award for Research Excellence (FARE)
• K99/R00, Pathway to Independence Award
• NIGMS Postdoctoral Research Associate (PRAT) Program
• National Research Service Award (NRSA), individual predoctoral and postdoctoral fellowships
• Intramural Research Fellowships for NICHD postdoctoral and clinical fellows

TEACHING
• Skills workshops on curriculum design and active learning
• Six-week college teaching workshop series, each summer
• “Becoming an Effective Scientist” course for postbac fellows; an eleven-week lecture and journal club series taught by postdocs and clinical fellows

CAREERS
• Job interview preparation
• Alumni speakers and panels from various career paths
• Editing of job application materials (c.v., cover letter, resume, teaching/research statement)
• Guidance for IDPs/Annual Progress Reviews
• One-on-one advising and career counseling

ANNUAL FELLOWS RETREAT
• This year’s retreat will be held Spring 2021
• Postdocs, clinical fellows, graduate students, resident
• Off campus, one day event
• Current scientific topics, keynote addresses, fellow talks, posters, networking, and career events

SCIENCE COMMUNICATION
• Presentation skills (oral, poster, and chalk talks)
• Annual Three-Minute Talks (TmT) Competition
• Graduate student talks
• Individual sessions with a public speaking coach

WRITING AND PUBLISHING
• Editorial service
• Grant preparation
• Online classes for scientific writing
• Getting published: strategies and approaches

MEDICAL AND GRAD SCHOOL PREPARATION
• Individual consulting
• Mock interviews
• Shadowing opportunities

For more information and resources, visit fellows.nichd.nih.gov

Any questions? Contact:
Dr. Erin Walsh
Acting Director, Office of Education, NICHD, NIH
erin.walsh@nih.gov | 301-435-1104

LEFT TO RIGHT: Erin Walsh, PhD; Triesta Fowler, MD; Fady Hannah-Shmouni, MD

LEFT TO RIGHT: Carol Carnahan, BS; Monica Cooper, BA
The database of NICHD alumni from 2008 to the present continues to be updated.

We compiled a valuable list of organizations that accept grant applications from NIH intramural fellows, through both NIH and non–NIH funding mechanisms. It can be found on the NICHD fellows’ wiki site. For NICHD, 18 Fellows Award for Research Excellence (FARE) awards were made for the 2021 competition.

The Office of Education also worked collaboratively with both administrative and scientific groups within DIR to achieve overall training goals and develop policies on various topics, such as tenure track mentoring and Affinity Group Seminar Series for invited speakers.

The Fellows Intramural Grants Supplement (FIGS) program continues to recognize and stimulate grant applications among fellows, and we launched the fourth cycle for our Intramural Research Fellowships (IRFs), a competitive internal funding opportunity for NICHD postdoctoral and clinical fellows. Its ultimate goal is to promote fellows’ grant writing and to enhance their awareness of various components for an NIH grant application.

The Institute continues its exchange program with INSERM (the Institut National de la Santé et de la Recherche Médicale) in France, which provides a unique opportunity for US and French scientists to obtain postdoctoral training with French and US mentors, respectively, and, since 2016, up to three second- and third-year medical students from Santa Casa de São Paulo School of Medical Sciences (Brazil) train with NICHD investigators for the Future Researchers Program. We are also actively working on a new partnership with Howard University, Washington, DC, for research, training, and mentoring opportunities.

The Fellows Recruitment Incentive Award (FRIA) continues to support investigators who recruit postdocs from populations traditionally under-represented in science, and these investigators also exhibit superior mentoring ability and demonstrate a commitment to the career development and academic progress of their fellows.

The alumni group for our NICHD Developing Talent Scholars Program, in its 10th year, is now at 19 individuals, and four new postbaccalaureate fellows joined the program in 2020. The Developing Scholars program focuses on providing trainees with a customized academic and professional development plan, which includes exposure to intramural activities and resources as well as supporting their career advancement.

We developed a new Online Fellows Exit Survey, which will be released soon for our trainee population. It aims to obtain a comprehensive view of trainees’ experiences at NICHD, to stay connected with the trainees, and learn of their career moves, as well as to help us improve our training program.

Postdoctoral fellows will be given the opportunity to organize and teach our annual course for postbaccalaureate trainees, which will be entering its 15th year for spring of 2021.

The Office of Education is fully committed to and actively involved in graduate and professional school advising and career counseling for all of our fellows, as well as to provide fellows with key resources to explore various scientific careers, help them set achievable goals, and build their professional networks.
The 16th Annual Fellows Meeting was postponed until 2021 as a result of the COVID-19 pandemic. Each spring, this retreat is held for about 120 trainees to address scientific developments and careers and includes presentations by fellows, career panel sessions, and a poster presentation by each attendee. The program is developed and run by a fellows' steering committee.

The NICHD Connection monthly newsletter continues its focus on mentoring, careers, and academic programs for young scientists, publishing its 126th issue in November 2020 and reaching all members of the intramural division and our alumni.

The office welcomed new staff members and Erin Walsh became Deputy Director of the Office. Triesta Fowler took on a new role in the Office of Education as Director of Communications and Outreach and Fady Hannah-Shmouni as the Director of Graduate Medical Education, and Monica Cooper joined the Office of Education as a Program Specialist.

Contact
For further information, contact Dr. Erin Walsh (erin.walsh@nih.gov).
The NICHD intramural clinical research program currently includes 60 protocols with six main areas of focus: (1) adult, pediatric, and reproductive endocrinology; (2) human genetics; (3) normal growth and development; (4) national/international public health; (5) women’s health; and (6) adolescent gynecology. The protocols are conducted by 27 NICHD Principal Investigators and 200 associate investigators. The NICHD clinical protocol portfolio spans the spectrum from Natural History to therapeutic trials. Eleven protocols involve an investigational drug or device. Four protocols support our teaching mission. Approximately half the protocols include pediatric patients.

In collaboration with Karim Calis from the Office of the Scientific Director, the NICHD Office of the Clinical Director assists NICHD investigators by sponsoring investigational new drugs. The NICHD Office of the Clinical Director (OCD) continues to support the NICHD Data and Safety Monitoring Committee (DSMC), which is chaired by Dr. Frank Pucino.

Contact
For more information, email fdporter@helix.nih.gov or visit https://www.nichd.nih.gov/about/org/dir/osd/mt/cd.
Clinical Trials at NICHD

Numerous clinical protocols are run by the NICHD, Division of Intramural Research (for a complete listing, please visit https://www.clinicaltrials.gov/ct/search?term=nichd). The following is a list of investigators within the DIR who recruit patients, and their contact information. For detailed information on all related research projects, please check the individual investigator’s listing in the report.

Bone and Matrix Biology in Development and Disease
  » Natural History Studies on children and adults with osteogenesis imperfecta, both dominant and recessive forms. Secondary features are a focus, including scoliosis, cardio-pulmonary and metabolic function, audiology and basilar invagination, as well as identification of causative genetic mutations. Patients may be referred to DR. JOAN MARINI at oidoc@helix.nih.gov.
  » Screening and diagnosis on patients with suspected connective tissue disorders, especially rare forms of osteogenesis imperfecta, other bone fragility disorders and melorheostosis. Patients and their families receive comprehensive evaluations, counseling, and risk assessment. Patients may be referred to DR. JOAN MARINI at oidoc@helix.nih.gov.

Developmental Endocrine Oncology and Genetics
  » Patient-oriented research into the etiology, pathophysiology, genetics, diagnosis, localization, and treatment of pheochromocytoma (PHEO) and paraganglioma (PGL). Patients may be referred to DR. KAREL PACAK at karel@mail.nih.gov or MARIANNE KNUE at marianne.knue@nih.gov or 301-827-3355.
  » Research on endocrine, genetic, and other pediatric disorders that are associated with the predisposition to endocrine and other tumors, abnormal development in fetal or later life and may affect the pituitary, the adrenal and other related organs. Patients may be referred to DR. CONSTANTINE STRATAKIS at stratakc@mail.nih.gov or to DR. ELENA BELYAVSKAYA at 301-496-0862.
  » Research investigating the causes, complications, and treatment of Primary Aldosteronism. Patients may be referred to DR. FADY HANNAH-SHMOUNI at fady.hannah-shmouni@nih.gov or DR. CRYSTAL KAMILARIS at crystal.kamilaris@nih.gov.
  » Research investigating the long-term effects of Cushing disease in childhood. Patients may be referred to DR. MEG KEIL at keilm@mail.nih.gov or 301-435-3391.
  » Study on the safety and efficacy of pegvisomant in children and adolescents with growth hormone excess, who have persistent disease after surgical and/or radiation treatment or are not eligible for those. Patients may be referred to DR. CONSTANTINE STRATAKIS at stratak@mail.nih.gov or to DR. CHRISTINA TATSI at 301-451-7170.
  » Studies into how genetics play a role in the development of obesity. Patients may be referred to DR. JACK YANOFSKI at yanovskj@mail.nih.gov or 301-435-8201.
  » Studies on pediatric disorders that are associated with the predisposition to develop obesity and diabetes including Bardet-Biedl Syndrome, Alström Syndrome, Prader-Willi Syndrome, leptin receptor deficiency, PCSK1 deficiency, and Pro-opiomelanocortin (POMC) deficiency. Patients may be referred to DR. JACK YANOFSKI at yanovskj@mail.nih.gov or 301-496-4168.
  » Pharmacotherapy of excessive hunger and obesity in patients with Prader-Willi syndrome, Bardet-Biedl syndrome, and other rare disorders with known genetic causes. Patients may be referred to DR. JACK YANOFSKI at yanovskj@mail.nih.gov or 301-496-6726.
» Evaluation of patients with endocrine disorders that are associated with excess androgen, including different forms of congenital adrenal hyperplasia. Patients may be referred to DR. DEBORAH MERKE at dmerke@nih.gov or DR. ASHWINI MALLAPPA at ashwini.mallappa@nih.gov or MS. LEE ANN KEENER at 240-858-9033 or leeann.keener@nih.gov.

» Studies on patients with genetic disorders related to altered cholesterol metabolism. This includes patients with Smith-Lemli-Opitz syndrome (SLOS) and Niemann-Pick Disease, type C (NPC). For NPC, patients may be referred to DR. FORBES PORTER at fdporter@mail.nih.gov, MS. NICOLE FARHAT at 301-594-1765 or MR. DEREK ALEXANDER at 301-827-0387. For SLOS, patients may be referred to DR. FORBES PORTER at fdporter@mail.nih.gov or MS. KISHA JENKINS at kisha.jenkins@nih.gov.

» Study of individuals with CLN3, or Juvenile Neuronal Ceroid-Lipofuscinos (Juvenile Batten Disease) and their family members. Interested participants may be referred to DR. AN NGOC DANG DO at an.dangdo@nih.gov or MS. KISHA JENKINS at 301-594-2005.

» Studies of patients with genetic disorders related to an abnormal function of the creatine transporter gene causing creatine transport deficiency (CTD). Patients may be referred to MR. JOHN PERREault at 301-827-9235 or to MS. KISHA JENKINS at 301-594-2005.

» Studies to identify novel genetic causes of idiopathic growth disorders using exome sequencing. Subjects will include children and adults with either short stature or tall stature without a known cause. Patients may be referred to DR. JEFFREY BARON at baronj@cc1.nichd.nih.gov or DR. YOUN HEE JEE at jeeyh@mail.nih.gov.

» Studies on metabolic effects of food additives (high intensity sweeteners) with special focus on pregnancy, and prenatal and infantile development. Interested participants may be referred to DR. KRISTINA ROTHER at 301-435-4639 or kristina.rother@nih.gov.

Maternal–Fetal Medicine, Imaging, and Behavioral Development

» **Studies with healthy subjects to test and calibrate non-invasive optical imaging technology for functional brain imaging.** The study is important to investigate the NIRS imaging system to explore techniques that will potentially improve the feasibility and reliability of the system according to the needs of the population whom existing imaging systems are unsuitable for. Functional near infrared spectroscopy (fNIRS) is an emerging non-invasive imaging technique to assess brain function. fNIRS measurements are based on the local changes in cerebral hemodynamic levels (oxy-hemoglobin and deoxy-hemoglobin) associated with brain activity. Due to neuro-vascular coupling, local changes in oxyhemoglobin and deoxyhemoglobin levels can serve as an indirect measure of brain activity. To probe changes in Oxy- and Deoxy-hemoglobin concentrations in the cortex that are caused by brain activity, different tasks such as the n-back, go-nogo tests will be administered to quantify spatial and temporal brain activity. Subjects may be referred to DR. AMIR GANDJBAKHCHE at gandjbaa@mail.nih.gov.

» **Mirror neuron network dysfunction as an early biomarker of neurodevelopmental disorder.** In this study, functional near-infrared spectroscopy (fNIRS) combined with electroencephalography (EEG) to measure brain activity in the mirror neuron network (MNN). The MNN is associated with the development of sophisticated social behaviors that emerge in typical infants. By modeling MNN development, we hope to uncover a sensitive measure of deviations in social communication development before clinical behavioral deficits can be detected. MNN activation has been indicated through mu rhythm suppression using EEG. The first part of the study involves adult subjects to determine whether MNN activation can be elicited, using a motor observation and a simultaneous execution paradigm using EEG/fNIRS systems. The synchronicity of these signals using more advanced machine learning methods to examine how the
features from both signals relate to each other and help characterize brain function in the mirror neuron network. In the next step, typically developing infants and infants at risk for developmental delays from 9–12 months of age are recruited. At-risk infants will be brought in again at 24 months of age to evaluate any deviations in their social communicative development. We will examine their developmental status at 24 months in relation to their initial neural data to determine whether MNN activation can predict developmental outcomes. For more information on the study, please contact DR. AMIR GANDJBAKHCHE at gandjbaa@mail.nih.gov.

» Biological Markers for the Prediction of the “great obstetrical syndromes”: A Longitudinal Study. This is a prospective cohort study of biomarkers in the great obstetrical syndromes to examine the natural history of normal pregnancy and the most frequent pregnancy complications. The goal is to develop sensitive, specific, and parsimonious predictive models to identify the patients at risk for developing complications of pregnancy using a combination of clinical and biological markers (biochemical and biophysical). For more information on the study, please contact DR. ROBERTO ROMERO at romeror@mail.nih.gov.

Pediatric and Adolescent Gynecology

Studies awaiting Institutional review Board approval (slated for December 2020):

» Data Collection Study of Pediatric and Adolescent Gynecology Conditions. This study is designed to perform deep phenotyping and data collection of children and adolescents presenting with gynecologic conditions including congenital anomalies. For additional information, contact DR. VERONICA GOMEZ-LOBO at veronica.gomez-lobo@nih.gov.

» Gonadal Tissue Freezing for Fertility Preservation in Girls at Risk for Ovarian Dysfunction and Primary Ovarian Insufficiency. This study is designed to evaluate possible mechanisms of follicle loss/dysfunction in children with Turner syndrome and classic galactosemia as well as adolescents with recent premature ovarian insufficiency. Ovarian tissue cryopreservation will be performed and a portion of the tissue will be stored for the patient's own use in the future. For additional information, contact DR. VERONICA GOMEZ-LOBO at veronica.gomez-lobo@nih.gov.

» Androgen Receptor, Implications for Health and Wellbeing: Natural History Study of Patients with Androgen Insensitivity. Research in individuals with the androgen receptor gene and consequently receptor abnormalities will allow better health care for these individuals and may also begin to elucidate possible androgen receptor mediated mechanisms for differences in physiology and health in other populations. For additional information, contact DR. VERONICA GOMEZ-LOBO at veronica.gomez-lobo@nih.gov.

Physical Biology and Medicine

» Studies on patients with genetic disorders related to fragile sarcolemma muscular dystrophy. This includes Limb-Girdle Muscular Dystrophy type (LGMD) 2B-F, I, L, Myoshi Myopathy (MM), Becker Muscular Dystrophy (BMD), Myoshi Muscular Dystrophy-3 (MMD3). Patients may be referred to DR. JOSHUA ZIMMERBERG at zimmerbj@mail.nih.gov or MS. HANG WATERS at watershn@mail.nih.gov.
Reproductive Endocrinology and Gynecology

» Research on reproductive disorders affecting the endometrium (such as recurrent implantation failure) using endometrial biopsy. Patients can contact DR. ALAN DECHERNEY at decherna@mail.nih.gov or 301-594-5494.

» Research on reproductive function in sickle cell disease. Patients can contact DR. ALAN DECHERNEY at decherna@mail.nih.gov or 301-594-5494.
The goal of the Fellowship is to train individuals to provide specialized patient care in Maternal-Fetal Medicine, as well as to prepare candidates for a career in academic medicine as physician scientists. It is a three-year training program. There is the possibility of completing a combined Maternal-Fetal Medicine and Human Genetics Fellowship, and candidates can opt to complete a PhD in the Department of Physiology at Wayne State University.

The 18-month clinical rotations include maternal-fetal medicine or high-risk obstetric service, obstetrical ultrasound, labor and delivery, intensive care unit, and elective rotations. The program is housed at Hutzel Women’s Hospital, Detroit, and Faculty Members are Wayne State University appointees as well as Attendings of the Detroit Medical Center (DMC) in Detroit, MI. The program is approved for seven positions, two of which are funded by the Perinatology Research Branch (PRB), which is also housed at the DMC, and the remainder by the DMC itself. The Fellowship emphasizes clinical, translational, and basic science research (18 months are dedicated to research). Ideal candidates for the Program are well-trained individuals from a university program who wish to pursue a career in academic medicine, thrive in a rigorous and challenging environment, and are goal-oriented and self-motivated.

The Fellowship stresses a multidisciplinary approach to the complications of pregnancy. There is a strong emphasis on prenatal diagnosis of congenital anomalies with ultrasound, and graduates are expected to be proficient in two-dimensional and three-dimensional ultrasound, fetal echocardiography, and advanced imaging techniques such as Doppler velocimetry, as well as ultrasound-guided invasive procedures such as amniocentesis. Opportunities for laboratory-based research and training are available at the PRB in the fields of parturition, reproductive immunology, placental pathology and biology, biomarker discovery, and systems biology in reproduction. Alternative opportunities are available at the C.S. Mott Center of Wayne State University.

The PRB’s primary areas of interest are the mechanisms responsible for obstetrical disease, prediction and prevention of preterm birth, prenatal diagnosis of congenital anomalies, the role of infection and inflammation in perinatal disease, fetal growth and development, placental pathology, and the use of high-dimensional biology techniques to identify biomarkers for preterm labor, preterm PROM (premature rupture of membranes), preeclampsia, fetal death, and IUGR (intrauterine growth restriction).

Detailed information about the training program is available at: [http://mfmfellowship.org](http://mfmfellowship.org). The website also contains information about the faculty and their publications and awards. The Director of the Fellowship Program is Dr. Lami Yeo. The Program is sponsored by the PRB, the DMC, and Wayne State University. Fellows are employees of the DMC, and program oversight is with the Office of Graduate Medical Education of the DMC.
NICHD–NIDDK–NIDCR Inter–Institute Endocrine Training Program

The Inter-Institute Endocrinology Training Program (IETP) is a three-year training program that seeks to train internal medicine physicians to become first-rate endocrinologists dedicated to investigative careers. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the National Institute of Child Health and Human Development (NICHD), and the National Institute of Dental and Craniofacial Research (NIDCR) participate in the program, with faculty from all three institutes.

Clinical and research training
Clinical training occurs largely in the first year. At any one time, fellows are responsible for five to ten patients on the inpatient service of the NIH. Under the supervision of the endocrine faculty, the trainee has complete responsibility for all aspects of a patient’s care. Fellows make daily rounds, discuss patients with the attending physicians, and participate in management decisions related to both patient care and clinical investigation. Although all patients are admitted under peer-reviewed research protocols, there are many other aspects of diagnosis and patient care that fall entirely under the discretion of the endocrine fellows.

Developing an independent career as a physician scientist is the primary focus during the second and third years of training; emphasis is placed on how to develop research questions and hypothesis-driven research protocols. To this end, the second and third years are spent primarily in the laboratory or conducting clinical research under the mentorship of a senior investigator in one of the several endocrinology branches of the NIH. During this research period, fellows continue to gain active clinical experience through bi-weekly continuity outpatient clinics (general endocrinology as well as diabetes clinics) and by participating in clinical conferences. In addition, fellows on the endocrine service serve as consultants to other services within the Clinical Center, where patients are not selected with regard to endocrine problems. Thus, fellows gain experience with the several common endocrine problems that may occur in any general medical ward. Clinical research activities include programs in all the areas of endocrine and metabolic disease. Study design, outcome measures, statistical analysis, and ethical and regulatory issues are stressed.
The IETP provides a comprehensive training experience that involves not only the NIH clinical branches working in endocrinology but also Georgetown University Hospital, Washington Hospital Center, and Walter Reed Medical Center. The basic and clinical endocrine research facilities at the NIH are among the most extensive and highly regarded in the world. Thus, the fellowship is ideal for physicians who seek a broad education in both research and clinical endocrinology.

Publications


Collaborators

- Kenneth Berman, MD, Director, Endocrine Training Program, Washington Hospital Center, Washington, DC
- Rebecca Brown, MD, Diabetes, Endocrinology, and Obesity Branch, NIDDK, Bethesda, MD
- Alan H. DeCherney, MD, Reproductive Endocrinology and Infertility Training Program, NICHD, Bethesda, MD
- Rachel Gafni, MD, Craniofacial and Skeletal Diseases Branch, NIDCR, Bethesda, MD
- Phillip Gorden, MD, Diabetes, Endocrinology, and Obesity Branch, NIDDK, Bethesda, MD
- James C. Reynolds, MD, Nuclear Medicine Department, NIH Clinical Center, Bethesda, MD
- William F. Simonds, MD, Metabolic Diseases Branch, NIDDK, Bethesda, MD
- Joseph Verbalis, MD, Director, Endocrine Training Program, Georgetown University Medical Center, Washington, DC
- Lee S. Weinstein, MD, Metabolic Diseases Branch, NIDDK, Bethesda, MD

Contact

For more information, email Ranganath.Muniyappa@nih.gov or go to [https://www.niddk.nih.gov/research-funding/at-niddk/training-employment/medical-student-md/inter-institute-endocrinology-fellowship-program](https://www.niddk.nih.gov/research-funding/at-niddk/training-employment/medical-student-md/inter-institute-endocrinology-fellowship-program).
Pediatric Endocrinology Inter-Institute Training Program

The Fellowship in Pediatric Endocrinology is a three-year, Accreditation Council for Graduate Medical Education (ACGME)–accredited program. Applicants must have completed a residency in Pediatrics or Medicine/Pediatrics and be eligible for the American Board of Pediatrics certification examination (exceptions can be made on an individual basis according to ACGME rules). Three fellowship positions are available per year. Training takes place predominately at the National Institutes of Health Clinical Center, which is one of the largest and most sophisticated research institutions in the United States. Additional clinical training takes place at Children’s Hospital in Washington, DC, and at Walter Reed National Military Medical Center in Bethesda, MD. The fellowship is designed to provide clinical and research exposure that fosters the development of academic pediatric endocrinologists with experience in clinical, translational, and/or basic research.

The URL https://www.nichd.nih.gov/about/org/dir/osd/tp/peitp provides more detailed information about the program.
Program structure

The Pediatric Endocrinology Fellowship at NIH consists of one year of clinical training and two years of combined clinical and research training.

First year. A typical training schedule for first-year fellows includes four months at the NIH Clinical Research Center, four months at Children’s National Health Systems (CNHS), two months at Walter Reed National Military Medical Center, one month on consult service, and one month elective (e.g., at The Johns Hopkins University Hospital, Baltimore, MD). Continuity clinics are held once a week and alternate between the NIH outpatient pediatric endocrine clinic and the diabetes and general endocrine outpatient clinics at CNHS. In addition, multi-disciplinary clinics in long-term follow-up for bone disorders, neuroendocrine tumors, disorders of sexual development, obesity, and other conditions are offered. The Clinical Center maintains clinical research protocols involving, among others, the treatment of adrenal and pituitary tumors, congenital adrenal hyperplasia, McCune-Albright syndrome, disorders of sexual development, obesity, and lipodystrophy.

Second and third years: During the second and third years, mandatory clinical responsibilities are limited to one half-day continuity clinic per week and inpatient pediatric endocrine consultation on an on-call basis for three months per year. Fellows learn how to develop a research protocol, conduct a clinical study or basic research, evaluate the results, and generate presentations and manuscripts suitable for publication. Fellows may choose to work in a laboratory setting, clinical setting, or both, and they perform state-of-the-art basic and clinical research closely supervised by internationally known mentors. During the first year, a research mentor is chosen and the fellow's progress is monitored by the Scholarship Oversight Committee. Many of our fellows choose academic careers following graduation.

Application information

Applications are submitted through ERAS (Electronic Residency Application Service). The application must contain three letters of reference, medical school transcripts, USMLE or COMLEX scores, a personal statement, and a CV. The program participates in the NRMP (National Resident Matching Program) match; pediatric endocrinology is now part of the fall subspecialty match. Applications must be submitted including all required information, and interviews are conducted from September through November. Applicants must register with both NRMP and ERAS (http://www.nrmp.org; https://www.aamc.org).

Collaborators

- Andrew Dauber, MD, MMSc, Director, Pediatric Endocrinology, Children’s National Hospital, Washington, DC
- Karen S. Vogt, MD, Program Director, Pediatric Endocrinology, Walter Reed Medical Center, Bethesda, MD

James Mills, MD, Senior Investigator, Division of Epidemiology, Statistics, and Prevention Research, NICHD
Constantine A. Stratakis, MD, D(med)Sci, Head, Section on Genetics & Endocrinology, NICHD
Jack Yanovski, MD, PhD, Head, Section on Growth and Obesity, NICHD
Rebecca J. Brown, MD, MHSc, Lasker Tenure-Track Investigator, Diabetes, Endocrinology, and Obesity Branch, NIDDK
Stephanie Chung, MD, Lasker Tenure-Track Investigator, Diabetes, Endocrinology, and Obesity Branch NIDDK
Rachel Gafni, MD, Senior Research Physician, Craniofacial and Skeletal Diseases Branch, NIDCR
Ellen Leschek, MD, Program Director, Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK
Deborah Merke, MD, Adjunct Investigator, NICHD & Chief, Section of Congenital Disorders, CC
Miranda Broadney, MD, MPH, Program Director, Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK
Brittney A. Corbin, BA, Pediatric Endocrine Fellowship Program Coordinator, NICHD
Contact
Kristina I. Rother, MD, MHSc, Program Director
Kristina.Rother@nih.gov
tel: 301-435-4639

Brittney Corbin, BA, Program Coordinator
Brittney.Corbin@nih.gov
tel: 301-496-4786

Pediatric Endocrinology Training Program
NICHD, NIH
Building 10, Room 2-5142
10 Center Drive
Bethesda, MD 20892-23330
NICHD Pediatric and Adolescent Gynecology Training Program

The Pediatric and Adolescent Gynecology (PAG) Fellowship training program is a two-year program, not accredited by the ACGME (Accreditation Council for Graduate Medical Education), comprising faculty from the National Institute of Child Health and Human Development (NICHD), Children’s National Health Systems (CNHS), and the MedStar Washington Hospital Center (MWHC). The mission of the fellowship program is to ensure that the graduate possesses the knowledge, skills, and professional attributes essential for the function as a consultant to pediatricians, family practitioners, obstetricians, and gynecologists for girls from birth up to age 18–21 years with pediatric gynecologic concerns, as well as for women born with congenital anomalies. Qualified candidates must be U.S. Citizens or Green Card holders and have completed an accredited residency in Obstetrics and Gynecology in the United States and be Board-eligible in this specialty.

Program structure
Currently, the fellowship alternates between recruiting one to two fellows per year. A schedule is established by which fellows rotate through general pediatric and adolescent gynecology clinics (including a heavy-menses clinic), and surgeries, specialty clinics (reproductive endocrine, Turner syndrome, PROUD [positive re-evaluation of urogenital differences clinics, or disorders of sex development] and vulvar dermatology clinics), colorectal surgery at CNHS, and research and consult/clinics at the NIH Clinical Center. The outpatient services do not depend on fellows for clinical care, which allows flexibility to maximize the educational experience. Surgeries are performed by the fellow on call with a resident, and the fellow functions as the surgical instructor during such cases. Complex surgeries are performed by both fellows as co-surgeons. Rotations, including one month in Child Abuse, are scheduled during the second year of fellowship. Electives in Adolescent Medicine (eating disorders), as well as travel to other sites for further complex anomaly training are available.

For successful completion of this training program, the fellow is required to design, implement, complete, describe, and report at least one research study. A second multi-center project must be designed and submitted, or collaboration with a project submitted to the Fellows Research Network at North American Society for Pediatric and
Adolescent Gynecology (NASPAG) is required. During the fellowship, fellows have thirty percent of their time protected for clinical research. The research takes place throughout the two years of training on 1.5 weekdays, except during outside rotations.

Structured training includes a series of introductory seminars geared to the first-year fellows, which take place from July to September in the first year. The introductory seminars provide a historical perspective and basic understanding of the practice of Pediatric and Adolescent Gynecology and Reproductive Endocrinology (REI), as well as of statistics. Fellows are required to attend a weekly colorectal conference, a monthly DSD (Differences of Sexual Development) clinic conference, a monthly Turner’s clinic conference, combined REI/PAG Journal Club/case conferences, lectures, and quarterly research meetings. In addition, fellows are encouraged to attend the monthly Reproductive Endocrine Journal club and DSD-TRN (Translational Research Network) case conference.

In addition to larger groups, mentors of individual laboratories to which the fellow is affiliated generally meet on a weekly basis to review research progress. Furthermore, fellows are encouraged to participate in the didactic training offered at national meetings, such as the NASPAG, the American Society for Reproductive Medicine, the Society for Gynecologic Investigation, and the Pediatric Endocrine Society. Moreover, fellows are encouraged to attend specialty meetings in their chosen interest areas, such as the Oncofertility Consortium and Differences in Sex Development–Androgen Insensitivity annual meetings.

Application Information
Applications are submitted using the common application form on NASPAG. They are accepted in July before the anticipated start date, and interviews are typically held in August or September before the start date. Selection of candidates is processed through the National Resident Matching Program (NRMP). One to two positions are available in alternating years.

Please visit the URL below for detailed program information:

Publications

**Contact**

Veronica Gomez-Lobo, MD, Program Director  
[veronica.gomez-lobo@nih.gov](mailto:veronica.gomez-lobo@nih.gov)  
301-435-7567

Sofia Getachew, MSPM, Program Coordinator  
[sofia.getachew@nih.gov](mailto:sofia.getachew@nih.gov)  
301-435-6926

Pediatric and Adolescent Gynecology Training Program  
NICHD, NIH  
10 Center Drive  
Building 10, Room 8N248, MSC 1840  
Bethesda, MD 20892-23330
Reproductive Endocrinology and Infertility Training Program

The Intramural NICHD Reproductive Endocrinology and Infertility Training Program sponsors a three-year clinical fellowship in Reproductive Endocrinology and Infertility, which is accredited by the Accreditation Council for Graduate Medical Education (ACGME). The objective of the graduate medical education program is to train clinicians to serve as researchers and future leaders in the field of reproductive endocrinology with a view toward advancing basic, translational, and clinical science in reproduction. The program was started in 1978 and has since trained over 90 physicians in reproductive endocrinology. Upon completion of the rigorous scientific, clinical, and surgical curriculum, fellows may apply to the American Board of Obstetrics and Gynecology for certification in the subspecialty of Reproductive Endocrinology. The Fellowship in Reproductive Endocrinology and Infertility is served by faculty from four institutions: the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), the Uniformed Services University of the Health Sciences (USUHS), Walter Reed National Military Medical Center (WRNMMC), and the Shady Grove Fertility Center. The program accepts civilian and military graduates of U.S. residencies in Obstetrics and Gynecology and has the mission to train reproductive endocrinology and infertility fellows who will serve as faculty in academic, government, and military institutions, and to establish and maintain high standards of training for students and residents in obstetrics and gynecology, so that they can provide evidence-based, cutting-edge treatments to couples with infertility. Program graduates have become assistant, associate, and tenured professors and departmental chairs.

Fellows in the program rotate on clinical services of NICHD-supported intramural graduate medical programs in Medical and Pediatric Endocrinology as well as in Medical Genetics. The clinical training is robust. Thus, within the unique environment of the NIH, fellows participate in evaluation and management of rare and challenging endocrine conditions on the NIH Reproductive Endocrine Teaching Service. Clinical and surgical rotations take place at WRNMMC and the Shady Grove Fertility Center. Research is strongly emphasized. Trainees in the program must complete a thesis project and may choose among any research laboratory in the Institute’s intramural research program. To complete the research project, fellows are
Didactic instruction

Structured training includes a series of introductory seminars geared to first-year fellows, which take place from July to September of the first year. The introductory seminars provide a historical perspective and basic understanding of the practice of Reproductive Endocrinology. In weekly NIH teaching-rounds conferences, fellows review and discuss challenging cases with faculty and fellows. In addition, all faculty and fellows of all years are expected to attend the weekly Pre-operative and Fellows' conferences. Fellows also attend weekly research conferences sponsored by the NICHD, and they present updates on thesis work at the weekly “Research in Progress Conference.” Core training objectives of the ACGME are covered in special NIH grand rounds and by courses at the NIH or the WRNMMC in Bethesda. NIH Endocrine Grand Rounds provide additional training in medical, pediatric, and reproductive endocrine conditions. Regular attendance at a monthly journal club is expected. Fellows regularly attend ART clinical meetings, during which management of patients pursuing ART is discussed and outcomes are reviewed. In addition to larger groups, mentors of individual laboratories to which the fellow is affiliated generally meet on a weekly basis to review research progress. Furthermore, fellows are encouraged to participate in didactic training offered at national meetings, such as those of the American Society for Reproductive Medicine, the Society for Reproductive Investigation, and the Society for the Study of Reproduction. Moreover, fellows are encouraged to attend specialty meetings in their chosen interest areas.
**Publications**


**Collaborators**

- William H. Catherino, MD, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Alicia Christy, MD, *Reproductive Health, Veterans Administration Central Office, Washington, DC*
- John M. Csokmay III, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Kate M. Devine, MD, *Shady Grove Fertility, Washington, DC*
- Nanette Rollene, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Rhiana D. Saunders, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Saioa Torrealday, MD, *Walter Reed National Military Medical Center, Bethesda, MD*
- Eric A. Widra, MD, *Shady Grove Fertility, Washington, DC*

**Contact**

Reproductive Endocrinology and Infertility Training Program

NICHD, NIH

Bldg. 10, Rm 8N248

10 Center Drive

Bethesda, MD 20892-1840

Phone: 301-402-2141

Fax: 301-451-2857

Program Director: Micah J. Hill, DO; hillmicah@mail.nih.gov

Program Coordinator: Marilyn Minor; minormar@mail.nih.gov

Online Application: [https://students-residents.aamc.org/training-residency-fellowship/applying-fellowships-eras](https://students-residents.aamc.org/training-residency-fellowship/applying-fellowships-eras)
The Administrative Management Branch (AMB) in the Division of Intramural Research (DIR), NICHD, assists in the planning and managing of a variety of administrative management projects. The AMB provides administrative oversight for adherence to rules and regulations and expertise in administrative services to ensure that the NICHD continues to move forward in its mission. The AMB staff are a key resource to NICHD’s Scientific Director in the management and overall planning for the DIR.

The senior leadership within the AMB works directly with the Scientific Director and Deputy Scientific Director in strategic planning and administrative oversight, and plays a key role in maximizing the resources available to the DIR. The leadership provides guidance in all aspects of administration, represents the DIR at various NICHD programs and NIH–wide committees as well as at focus groups concerned with administration.

The AMB administrative staff provide professional, technical, and administrative support in functional areas that further the mission of the DIR. These include budget and financial management, human resources, visas, travel, simplified acquisition, contract management and project officer support, safety and security, building and facilities management, timekeeping, program planning and evaluation, and general administrative services.

The AMB staff serve as a liaison between the laboratories and branches that they support and the many other entities at the NIH, such as the Office of the Scientific Director, NICHD; the Office of the Director, NICHD; the Office of Intramural Research, Office of the Director; the Fogarty International Center; Human Resources offices; the Office of Research Services, including the Division of International Services; the Office of Research Facilities; and the Technology Transfer Branch.

Contact
For more information, email franciek@mail.nih.gov or visit http://amb.nichd.nih.gov.
AMB Staff

Izet Beckwith, Administrative Officer
Antoinette Chavez, Administrative Officer
Gina Elmore, Administrative Officer – Budget
Dena Flipping, Administrative Officer
Michelle Hudson, Administrative Officer – Team Leader
Joy Johnson, Administrative Officer
Bonnie Lancey, Administrative Officer
Keta Lawson-Davis, Administrative Officer
Wanda Logan, Administrative Officer
Lakeasha Mingo, Administrative Officer – Team Leader
Charlene Patrick, Administrative Officer – Team Leader
Mia Pulley, Administrative Officer – Team Leader
Natacha Rene, Administrative Officer
Nancy Richman, Administrative Officer
Sylvia Robinson, Administrative Officer – Procurement Lead
Beverley Todd, Administrative Officer
Marlene Taulton, Administrative Officer

Brittany Corum, Administrative Technician
Barbara Hardy, Administrative Technician
Ashley Mason, Budget Analyst
Michael Anderson, Facilities Assistant
Tamika Morgan, Facilities Assistant
Steven Norris, Facilities Manager
Giulia Mason, Management Analyst
Moona Braxton, Procurement Technician
Jennifer Smith, Procurement Technician
Anju Vergheese, Program Analyst
John Burton, Purchasing Agent
Jax Chang, Purchasing Agent
Towanda Daniels, Purchasing Agent
Roshonna Davis, Purchasing Agent
William Davis, Purchasing Agent
Sherry Jones, Purchasing Agent
James Law, Purchasing Agent
David Shen, Purchasing Agent
Hanumanth Vishnuvajjala, Purchasing Agent
Research Animal Management Branch

The Research Animal Management Branch (RAMB) supports all animal use and research within the NICHD Division of Intramural Research.

In addition to providing animal research support and veterinary consultation to NICHD investigators, the Research Animal Management Branch (RAMB) represents the interests of the NICHD DIR on all aspects of animal research conducted within the Intramural Research Program (IRP).

RAMB operates and manages the Building 6B Shared Animal Facility (SAF), Suite 6C127 of the Ambulatory Care Research Facility (ACRF) Animal Facility, the Building 6 Shared Zebrafish facility (SZF), and the Building 49 Xenopus facility. The Division of Intramural Research (DIR) Animal Program and NICHD Animal Care Use Committee (ACUC) have oversight over these facilities with regard to animal use as well as over NICHD animals in the Porter Neurosciences Research Center (PNRC) SAF, and in Central Animal Facilities (CAF). The RAMB Aquatics Program also provides aquatics animal care and husbandry to several institutes and centers (ICs) within their satellite facilities across the NIH. NICHD is considered a leader in the field of aquatics research animal care and support at the NIH.

The Building 6B SAF houses over 11,000 cages of specific pathogen-free (SPF) rodents and provides lead institute support to the animal-research activities of three Institutes (NICHD, NEI, and NIAMS). In addition to traditional rodent research support, the program supports NICHD behavioral research studies with a dedicated technician. The NICHD ACRF Animal Facility supports the animal research activities of the NICHD with four animal rooms and two procedure rooms. The RAMB provides care and housing for rodent and aquatic species housed within this restricted-access, disease-free animal holding facility.

NICHD Aquatics are housed within the Building 6 SZF, ACRF Animal Facility, and Building 49. The Building 6 SZF supports NICHD and NHGRI with 15,000 two-liter tanks with a total capacity of over 330,000 zebrafish. The Xenopus satellite frog facility in Building 49 provides primary animal care and research support to over 300 tanks of Xenopus used by NICHD DIR researchers.
As part of the NIH, RAMB staff participate in the formulation of policies and procedures that impact the care and use of laboratory animals throughout the country. RAMB leads the effort for triennial re-certification by AAALAC International. RAMB staff and many animal-user investigators have been active contributors to the NIH Animal Research Advisory Committee (ARAC) and other trans-NIH committees.

**NICHD RAMB website**

http://www.animalcare.nichd.nih.gov

**Additional Funding**

- In addition to direct funding by the Intramural Research Programs of NICHD, the RAMB is also funded by facility users from other NIH Institutes and Centers.

**Contact**

For more information, email robin.kastenmayer@nih.gov or visit http://www.animalcare.nichd.nih.gov.
The goal of the Bioinformatics and Scientific Programming Core (BSPC) is to provide expert bioinformatics support to NICHD researchers, assisting at all stages from experimental design through multiple iterations of analysis to final manuscript preparation. In addition, we develop software tools that can be applied to a wide range of bioinformatics, genomics, and general data analysis, both at NICHD and in the larger international scientific community. We coordinate training for staff and trainees in basic programming and genomic analyses to help build bioinformatics support directly within labs.

Structure
The BSPC uses a “hub and spoke” model, consisting of a central core of staff (currently in Building 10) coordinating with embedded bioinformaticians (currently in Buildings 6 and 49) working directly in laboratories. This allows us to build centralized infrastructure that can be re-used across many research programs, while at the same time maintaining focused and custom local support in labs. Joint meetings and discussion allow everyone, central and embedded, to share lessons learned and identify new tools and methods.

Projects overview
In 2020, the BSPC worked on 65 projects, collaborating with PIs, fellows, staff scientists, and staff clinicians across 29 laboratories. Of these, 30 were new projects and 35 were carried over from the previous year. The projects included assays such as bulk RNA-seq, single-cell RNA-seq, ChIP-seq, whole-exome sequencing, whole-genome sequencing, DNA methylation, CUT&RUN, bulk ATAC-seq, and single-cell ATAC seq. New projects this year also included assessment of protein motifs, identification of co-evolving protein domains, SLAM-seq, and structural variant calling. Some projects involved custom algorithm development and tool development, and many projects required integration with published studies. Two new projects converted legacy code used in labs into a modern software architecture, which the BSPC can maintain into the future. Roughly a third of the projects involved RNA-seq differential expression analysis.

Projects often begin with an in-depth discussion with researchers to understand the background and goals of the project. It is important
for us to understand the underlying biology and details of the experimental design (when applicable) for each project, so that we can make the most informed analysis decisions. We then provide a prioritized plan for the first round of analysis and schedule the work. There are often several iterations of analysis as a project progresses. Each iteration may add more sophisticated analyses, new data generated by the lab, or integrate results with published data. As expected for a no-cost shared resource, the time it takes for one iteration on one project is highly dependent on the existing workload across all other projects that we are handling in the Institute.

After each iteration, we meet to discuss the results in detail. The meeting includes a walk-through of the results, the computational background, discussion of how to use and interpret the tables, figures, and other output, and recommendations for next steps. Depending on the researchers’ interests, this can also include a discussion of the code and help with running it or adapting it to other projects in the lab. The next iteration of analysis is then planned, prioritized, and scheduled.

The BSPC’s collaboration includes writing the manuscript, producing figures and tables, consulting on interpretation, writing detailed computational methods, submitting data to public repositories, reviewing code, and submitting code to public repositories along with the complete software environments required to make the analyses reproducible.

Projects: computation and code
Most projects are multi-week or multi-month projects, which continue after many iterations and often require authoring substantial amounts of custom R and Python code. We work closely with NICHD’s Molecular Genomics Core, where much of the raw high-throughput sequencing data for NICHD are generated. We can access these data directly, avoiding the need to coordinate data transfer and/or storage space with researchers. Analysis performed by the BSPC makes extensive use of NIH’s Biowulf high-performance computing cluster, and there is no direct cost to researchers for work done by the BSPC.

To ensure long-term computational reproducibility, we build a complete software environment for each project, which allows us to track all versions of software and dependencies, and any one project’s environment can be updated without affecting any others. All source code is kept under version control so that the entire history of the project can be tracked. We also build reproducible workflows for each project that keep track of which results have been updated and, wherever possible, provide output as standalone, interactive HTML files, so that researchers can easily explore their results.

Additional software development and computational resources
The BSPC continues to develop and maintain publicly available open-source tools. One example is lcdb-wf, a system of workflows and pipelines to process high-throughput sequencing data, run extensive quality control, and perform differential ChIP-seq or RNA-seq analyses and which runs on NIH’s Biowulf computing cluster. We also continue to contribute to the Bioconda project, a system used by bioinformaticians worldwide to easily install biology-related software tools.

The BSPC maintains an RStudio Connect Server instance, which allows us to publish interactive applications that researchers can use to interactively explore and plot their data. We also maintain a GitLab instance in NICHD’s data center, which provides source code version control, issue tracking, and documentation for
projects we work on in such a way that they can be shared with collaborators. These repositories currently store tens of thousands of lines of Python and R code and documentation written by the BSPC and used in various projects.

**Publications**


**Collaborators**

- William J. Pavan, PhD, *Genomics, Development and Disease Section, NHGRI, Bethesda, MD*
- Michael E. Ward, MD, PhD, *Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda, MD*

**Contact**

For more information, email ryan.dale@nih.gov or visit https://www.nichd.nih.gov/about/org/dir/other-facilities/cores/bioinformatics.
Research Informatics Support for NICHD's Division of Intramural Research

The Computer Support Services Core (CSSC) Facility provides informatics and research services to intramural investigators of the Division of Intramural Research (DIR), NICHD, in five key areas: core IT support, clinical informatics, custom software development for scientific and administrative support, and biological visualization services.

Core IT Services
The CSSC continued to expand its services to the Division of Intramural Research (DIR) community in the following core IT areas:

NETWORK AND DESKTOP SERVICES
During the past year, the CSSC continued supporting reliable, secure, and efficient information technology solutions. This includes acquisition, maintenance, and support for licensed software used by the DIR community e.g., GraphPad Prism, Amira, DNASTAR Lasergene, MathWorks MATLAB, SnapGene, PyMol, and FlowJo, as well as network services (email, data backups, VPN, wireless configurations) and cross-platform desktop, server and application hosting in the Rock Spring and Bldg. 35 Data Centers. We also assist users in identifying, researching, and purchasing custom hardware configurations to match their research instrument requirements.

DATA-RECOVERY SERVICES
The CSSC implemented core data recovery tools for all media: hard drive, SSD, and flash etc., including RAID 0 and 5 recovery tools. Since 2005, the Core has recovered over five terabytes of research data from failed drives and media, saving many thousands of dollars in recovery costs.

Clinical informatics
The CSSC continued to support and develop applications related to clinical and translational medicine, including the Clinical Trials Database (CTDB) project. Such informatics tools allow researchers to design, collect, and report clinical observations related to natural history and interval-based studies. The total number of protocols and research projects supported by the CTDB team increased to 660 for 15 NIH institutes, with an expansion of research questions to over 240,000. Since its inception, data from CTDB supported 1,500 NICHD publications.
Our software development group completed a release that included new features for Multi-language functionality, Dashboard integration within the interface, and Adverse Events management feature. The CTDB release included improvement in the functionality of data entry and bio-specimen and quality assurance modules. We also supported the Clinical Trial Survey System (CTSS), an application for patient self-reporting, servicing 81 active protocols. Implementation of the electronic consenting in CTSS positioned the researchers at NIMH to launch three COVID studies in record time.

The database development and reporting team implemented Oracle standby databases and Goldengate replication services to give the CTDB additional redundancy in the case of system outages. The team implemented an integration between CTDB and the NIMH Imaging database. We continued to support data-marts as new reporting requirements emerge, and to migrate data as needed. Additionally, the team worked closely with various PIs across the institutes to provide both management and research-related reports for clinical studies, publishing over 400 reports in the past year. The team applies the latest patches to all production database environments to ensure continued uninterrupted services, and monitors the successful completion of backup and data-mart transformation services.

**Custom software development for scientific and administrative support**

The CSSC provides custom software development for the DIR's scientific and administrative community.

We continued to enhance the Manuscript Tracking System (MTrac), a web-based application that automates the clearance and approval process for manuscripts in the DIR.

The DIRweb application supports several activities: the NICHD annual report, PI and Fellows retreats, Training tracking, Fellows' progress report, and Administrative Management Branch (AMB) personnel and travel package tracking.

DIRweb includes lab training web services for the NIH Enterprise Directory and Division of Occupational Health and Safety Training.

The team continued to release enhancements to the Fellows Annual Progress Report, a unified means for tracking and mentoring intramural trainees as well as for easing the re-appointment process. This solution provided the Office of Education with useful metrics regarding mentoring and training programs. We also updated the Exit Survey feature, a short survey allowing DIR Fellows a platform for providing feedback.

We continued to develop the new Package Tracking module for the DIR AMB, providing AMB staff real-time accuracy metrics for personnel and travel package compilation. We developed a feedback system to support real-time customer satisfaction collection for the AMB, Office of the Clinical Director, and laboratory administrative support staff. The system also offers more detailed feedback submissions periodically along with comprehensive response metrics.
The CSSC team continued to work on Cost Tracker, an application that permits capturing, organizing, and reporting of various expenses on a per-protocol basis. The work is done closely with the Office of the Clinical Director to improve protocol cost vs. effectiveness and a protocol-cost estimator module.

The CSSC team continues to develop and support several feedback systems to support real-time customer satisfaction collection. These include surveys for the AMB, Office of the Clinical Director, laboratory administrative support staff, and NICHDs Administrative Services Branch. The system also offers more detailed feedback submissions periodically along with comprehensive response metrics.

**Biological visualization services**

The CSSC team provided DIR laboratories with scientific communications and media services, including publication support, website support, audio/visual production, and print media.

The services were provided to: The NICHD DIR Annual Report, the DIR Annual Fellows and Scientific Retreats, the DIR Annual Fellows and Scientific Retreats, the Anita B. Roberts Lecture Series, the annual Mortimer B. Lipsett Lecture, the NICHD Exchange lecture series, NICHD research labs, and medical training programs, including the Pediatric Endocrinology Inter-Institute Training Program and the NICHD-NIDDK-NIDCR Inter-Institute Endocrine Training Program.

We supported the NICHD Office of Education by producing a monthly newsletter, “The NICHD Connection,” in collaboration with Intramural Fellows. We created print collateral for conferences, including the NICHD Scientific Retreat and NICHD Fellows Retreat, and updated recruitment materials for NICHD's clinical training programs. We also continued maintaining websites for the NICHD DIR Annual Report and Annual Fellows Retreat.

The CSSC continued to provide a platform for conducting scientific review by the Board of Scientific Counselors, administrative intranet support, and business operations. In 2020, we continued supporting the migration of the public facing laboratory websites from Confluence wiki to Drupal. The plan for Science@NICHD ([http://science.nichd.nih.gov](http://science.nichd.nih.gov)) is to keep hosting NICHD DIR internal sites.

**Additional Funding**

- The Clinical Trials Database (CTDB) project receives funding from other NIH Intramural Institute or Center programs, including NINR, NIMH, NIDCR, NIEHS, NIAMS, NINDS, CC, NHLBI, NIDCD, NHGRI, NCCIH, NIMHD, and NIDDK.

**Collaborators**

- Richard Childs, MD, *Clinical Director, NHLBI, Bethesda, MD*
- Maryland Pao, MD, *Clinical Director, NIMH, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, Clinical Director, NICHD, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

**Contact**

For more information, email ryan.dale@nih.gov.
MICROSCOPY AND IMAGING CORE FACILITY

NICHD Microscopy and Imaging Core

The mission of the NICHD Microscopy and Imaging Core (MIC) is to provide service in four different areas: (1) sample preparation for light and electron microscopy studies; (2) wide-field and confocal light microscopy; (3) transmission electron microscopy (TEM); and (4) image analysis and data extraction. The Facility is operated as a 'one-stop shop,' where investigators can, with minimum effort, go from their scientific question to the final data.

Mode of operation

Located on the ground floor of the Porter Building (building 35A), the MIC is accessible 24/7, and users can reserve time on each microscope by using an online calendar (https://nichd.agendo.science). The Facility is available free of charge to all NICHD investigators and, resources allowing, to anyone within the Porter building. The Facility is supported by the Office of the Scientific Director.

Vincent Schram is the point person for light microscopy and data analysis. The EM branch of the Facility is staffed by Chip Dye, and Lynne Holtzclaw is in charge of sample preparation (histology). Chip Dye and Lynne Holtzclaw report to Vincent Schram, who serves as acting director under the management of Chris McBain (NICHD). Tamás Balla (NICHD) acts as scientific advisor for the Facility.

The MIC has an open-door policy with the NINDS Light Imaging Facility (LIF, Building 35), where the two cores freely exchange users, equipment, and support. Although not officially sanctioned, this mode of operation provides extended support hours, wider expertise, and access to more equipment than each Institute could afford on its own. The MIC serves over 300 registered users in 68 laboratories. NICHD uses 80% of the Facility resources, NINDS 15%, and other Institutes (NIBIB, NIA, and NIMH) the remaining 5%.

Light microscopy

The MIC is equipped with six confocal microscopes, each optimized for certain applications:

1. Zeiss LSM 710 inverted for high-resolution confocal imaging;
2. Zeiss LSM 780 with a spectral detector;
3. Nikon Spinning Disk/Total Internal Reflection Fluorescence (TIRF)
hybrid microscope for high-speed confocal imaging or recording of membrane-bound events in live cells (TIRF);
4. Zeiss LSM 880 2-photon confocal for thick tissues and live animals;
5. Zeiss 800 optimized for advanced tiling experiments;
6. Zeiss 880 AiryScan with higher spatial resolution.

Several conventional (wide-field) light microscopes provide imaging modalities such as transmission (visible stains), large-scale tiling of tissue slices, high-speed phase contrast and differential interference contrast (DIC), and large specimens.

After an initial orientation, during which the staff research the project and decide on the best approach, users receive hands-on training on the equipment and/or software best suited to their goals, followed by continuous support when required. Once image acquisition is complete, the staff devise solutions and train users on how to extract usable data from their images.

**Electron microscopy**
The electron microscopy branch of the Facility processes specimens from start to finish: fixation, embedding, semi-thin and ultra-thin sectioning, staining, and imaging on the JEOL 1400 transmission electron microscope. Because of the labor involved, the volume is necessarily smaller than for the light microscopy branch, where end users do their own processing. In the past 12 months, Chip Dye processed a total of 145 samples for morphology studies.

**Tissue preparation**
Lynne Holtzclaw provided sample processing training and services for light and electron microscopy. Thirteen users were trained in-person in rodent perfusion, cryopreservation, cryosectioning, immunofluorescence and tissue clearing. With the assistance of Sara Felsen, Lynne Holtzclaw also dedicated a significant amount of time to bring RNAScope methodologies to the Core, allowing users to conduct investigations in the histology suite. NICHD users include Tamás Balla, Peter Basser, Sergey Bezrukov, Andres Buonanno, Leonid Chernomordik, Robert Crouch, Douglas Fields, Dax Hoffman, David Klein, Claire Le Pichon, Chris McBain, Karl Pfeifer, Forbes Porter, Dan Sackett, Yun-Bo Shi, Stanko Stojilkovic, and Marc Stopfer. She also provided assistance to the laboratories of Miguel Holmgren, Katherine Roche, Richard Youle, and Michael Ward (NINDS); Dietmar Plenz (NIMH); and Ellen Sidransky (NHGRI).

A collaboration with the laboratory of Richard Youle to study the accumulation of ubiquitinated protein aggregates in the brain and liver of a TAX1BP1 knock-out mouse was completed. We initiated a developmental rat pineal study, in collaboration with David Klein, with plans to probe samples using the RNAScope methodology. Lynne Holtzclaw is also working with the McBain Lab to investigate, via RNAScope, the expression of a potassium channel subunit in human parvalbumin (PV) interneurons.

**Image analysis**
High-end computer workstations with imaging software (Zeiss Zen, Nikon Element, Bitplane Imaris, SVI Hyugens and ImageJ) are also available.

Image processing based on neural networks (Artificial Intelligence or AI) is fast becoming a remarkably
powerful tool for image restoration, segmentation, and resolution improvement. The MIC has been actively looking at AI-powered solutions for image restoration and segmentation. The Nikon NIS-AI suite, an advanced software for noise removal and segmentation not possible with conventional methods, was purchased and is now available in the core.

Collaborators

• Tamás Balla, PhD, Section on Molecular Signal Transduction, NICHD, Bethesda, MD
• David C. Klein, PhD, Scientist Emeritus, NICHD, Bethesda, MD
• Carolyn L. Smith, PhD, Light Imaging Facility, NINDS, Bethesda, MD
• Richard J. Youle, PhD, Biochemistry Section, NINDS, Bethesda, MD

Contact

For more information, email schramv@mail.nih.gov or visit http://mic.nih.gov.
Molecular Genomics Core Facility

With the goal of understanding the genetic changes and mechanisms underlying human diseases, the Molecular Genomics Core (MGC) Facility supports NICHD investigators by providing next-generation deep sequencing and project data analysis.

Next-Generation sequencing and bioinformatics support
The MGC provides DNA and RNA sequencing services for genomic and genetic research to investigators within the NICHD. The MGC is currently operating with five sequencing machines. Most of our work is conducted on our high-capacity, production-scale machine: an Illumina HiSeq 2500. To increase the throughput of the HiSeq 2500 even further, we have a cBot liquid handler for automated high-precision loading of sequencing chips. Our most recent acquisition is an Illumina NovaSeq 6000, which has a much higher capacity than the HiSeq 2500. Two of the other sequencers, an Illumina MiSeq and an Ion Torrent Personal Genomics Machine, are smaller, faster machines, which can generate longer sequence reads of up to 400 base pairs. The fifth sequencer, is a Pacific Biosciences (PacBio) Sequel, which can sequence long single molecules of more than 100,000 base pairs. Our array of sequencers provides a suite of scales and capabilities. Our sequencing services include whole-genome, whole-exome, targeted exome, and gene-specific DNA sequencing, as well as whole-transcriptome sequencing (RNA-Seq), microRNA sequencing, microbiome sequencing, bisulfite sequencing (DNA methylome), ChIP-Seq, and ribosomal profiling. The PacBio Sequel permits mutation phasing, structural variant analysis, transposon location identification, and other analyses that are not possible or practical with the other sequencers.

Recently, the MGC acquired a 10X Genomics Chromium Single Cell Controller. The Chromium converts a suspension of single cells into cDNA libraries that are barcoded by cell of origin. The cDNAs can then be converted into sequenceable libraries and run on our Illumina HiSeq 2500 or NovaSeq 6000 to generate thousands of cell-specific transcriptomes.

The MGC provides significant primary data-processing and downstream bioinformatic support and can assist in designing...
experiments or sequencing strategies (for example, optimization of targeted exome design). During the past year, the MGC provided sequencing for 153 projects (2,509 samples) across the full spectrum of sequencing types, generating 6,205 gigabases of sequence; the projects involved 38 NICHD Principal Investigators from 11 Affinity Groups. In addition to sequencing and providing our standard primary analysis of the resulting data, the MGC delivered enhanced bioinformatic support to 24 NICHD investigators across nine Affinity Groups. Our mission is to offer accurate and innovative sequencing and bioinformatic tools to facilitate research into the diagnosis, counseling, and treatment of hereditary disorders, and to support basic research that promotes understanding of human health and development.

Publications

Collaborators
- Jeffrey Baron, MD, *Section on Growth and Development, NICHD, Bethesda, MD*
- Diana Bianci, MD, *Prenatal Genomics & Therapy Section, NHGRI, Bethesda, MD*
- Juan Bonifacino, PhD, *Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- Michael Cashel, MD, PhD, *Section on Molecular Regulation, NICHD, Bethesda, MD*
- Ajay Chitnis, MBBS, PhD, *Section on Neural Developmental Dynamics, NICHD, Bethesda, MD*
- David J. Clark, PhD, *Section on Chromatin & Gene Expression, NICHD, Bethesda, MD*
- Robert J. Crouch, PhD, *Section on the Formation of RNA, NICHD, Bethesda, MD*
- Mary Dasso, PhD, *Section on Cell Cycle Regulation, NICHD, Bethesda, MD*
- Katie Drerup, PhD, *Unit on Neuronal Cell Biology, NICHD, Bethesda, MD*
- Maria L. Dufau, PhD, *Section on Molecular Endocrinology, NICHD, Bethesda, MD*
- Benjamin Feldman, PhD, *Zebrafish Core, NICHD, Bethesda, MD*
- Marc Ferrer, MD, *3-D Tissue Bioprinting Laboratory, NCATS, Rockville, MD*
- Judith Kassis, PhD, *Section on Gene Expression, NICHD, Bethesda, MD*
- David Klein, PhD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Sergey Leikin, PhD, *Section on Physical Biochemistry, NICHD, Bethesda, MD*
- Claire E. Le Pichon, PhD, *Unit on the Development of Neurodegeneration, NICHD, Bethesda, MD*
• Henry L. Levin, PhD, Section on Eukaryotic Transposable Elements, NICHD, Bethesda, MD
• Mary Lilly, PhD, Section on Gamete Development, NICHD, Bethesda, MD
• Paul Love, MD, PhD, Section on Cellular and Developmental Biology, NICHD, Bethesda, MD
• Todd Macfarlan, PhD, Unit on Mammalian Epigenome Reprogramming, NICHD, Bethesda, MD
• Matthias Machner, PhD, Section on Microbial Pathogenesis, NICHD, Bethesda, MD
• Richard Marais, MD, Section on Molecular and Cellular Biology, NICHD, Bethesda, MD
• Joan C. Marini, MD, PhD, Section on Heritable Disorders of Bone & Extracellular Matrix, NICHD, Bethesda, MD
• Deborah Merke, MD, Section on Congenital Disorders, NICHD, Bethesda, MD
• Keiko Ozato, PhD, Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD
• Timothy J. Petros, PhD, Unit on Cellular and Molecular Neurodevelopment, NICHD, Bethesda, MD
• Karl Pfeifer, PhD, Section on Epigenetics, NICHD, Bethesda, MD
• Forbes D. Porter, MD, PhD, Section on Molecular Dysmorphology, NICHD, Bethesda, MD
• Pedro Rocha, PhD, Unit on Genome Structure and Regulation, NICHD, Bethesda, MD
• Dan Sackett, PhD, Division of Basic and Translational Biophysics, NICHD, Bethesda, MD
• Mihaela Serpe, PhD, Section on Cellular Communication, NICHD, Bethesda, MD
• Stanko S. Stojilkovic, PhD, Section on Cellular Signaling, NICHD, Bethesda, MD
• Yun-Bo Shi, PhD, Section on Molecular Morphogenesis, NICHD, Bethesda, MD
• Gisela Storz, PhD, Section on Environmental Gene Regulation, NICHD, Bethesda, MD
• Constantine Stratakis, MD, D(med)Sci, Section on Endocrinology and Genetics, NICHD, Bethesda, MD
• Michael E. Ward, MD, PhD, 3-D Tissue Bioprinting Laboratory, NINDS, Bethesda, MD
• Brant Weinstein, PhD, Section on Vertebrate Organogenesis, NICHD, Bethesda, MD
• Jack Yanovski, MD, PhD, Section on Growth and Obesity, NICHD, Bethesda, MD

Contact
For more information, email fdporter@mail.nih.gov or visit https://www.nichd.nih.gov/about/org/dir/osd/cf/mgl.
The NICHD Zebrafish Core

The NICHD Zebrafish Core was established in 2012 with the goal of providing its clients with consultation, access to equipment and reagents, and service in the area of zebrafish genetics. NICHD investigators as well as investigators from other NIH institutes and from outside the NIH are its clientele. The oversight committee for the Core comprises Harold Burgess, Ajay Chitnis, Brant Weinstein, and Katie Drerup. The Core’s activities consist of (1) oversight and support of client-specific projects, (2) custom generation of genetic zebrafish models, (3) troubleshooting of new methodologies with promising application in zebrafish, (4) maintenance and improvement of equipment and infrastructure, and (5) service and educational outreach.

Oversight and support of client-specific projects
Over 2019–2020, the Core engaged in research projects with ten labs and other customers and with two cores.

GENETIC DISSECTION AND CREATION OF HUMAN DISEASE MODELS OF STEROL METABOLISM (PORTER LAB, NICHD)
In previous years the Core used CRISPR-Cas9 technology to create genetic mutant zebrafish lines for the Porter lab in five genes: dhcr7, npc1, npc2, cln3 and ebp, which play roles in various steps of cholesterol metabolism. In 2018 phenotypes of npc1 mutants were characterized and published, and during the current year, a re-submission of a paper on npc2 phenotypes is currently being re-reviewed.

FUNCTION OF ZEBRAFISH ORTHOLOGS TO HUMAN GENES IMPLICATED IN DISORDERS OF THE PITUITARY-ADRENAL AXIS (STRATAKIS LAB, NICHD)
In previous years, the Core used CRISPR-Cas9 technology to generate zebrafish carrying loss-of-function mutations in four zebrafish orthologs to human genes, implicated by the Stratakis lab in human growth anomalies, and eight zebrafish orthologs to human adrenal hyperplasia and Cushing's disease–associated genes. A manuscript summarizing the characterization of one of these genes, gpr101, was prepared and submitted this year, and reviewer comments are being addressed prior to re-submission. Over the past several months, a new project was initiated to create a precisely edited zebrafish satb1 mutant line with a non-synonymous amino acid substitution that is cognate to a human disease-associated mutation of interest.
ASSESSING FUNCTIONS OF THE TTP FAMILY OF RNA–BINDING PROTEINS IN ZEBRAFISH
(BLACKSHEAR LAB, NIEHS)
The Core has been engaged over the past several years to use CRISPR-Cas9 technology to generate zebrafish carrying loss-of-function mutations in seven zebrafish TTP family orthologs and assist with preliminary phenotype characterization. Phenotypes for fourteen mutant alleles for all seven genes have proven to be weak or absent. RNAseq–based comparisons of the transcriptomes of mutants with control siblings was performed for six of the seven genes, revealing high and opposite sex-specific expression for two of the genes. Current plans are to independently repeat these findings and to intercross two lines to make double-nulls and thus potentially reveal phenotypes that were masked by functional redundancy.

FUNCTION OF ZEBRAFISH rca2.1 (KEMPER LAB, NHLBI)
The Kemper lab is interested in zebrafish rca2.1’s function, because it has certain similarities to human CD46 (a transmembrane glycoprotein that plays a role in regulating the complement system) not found in the mouse genome. We initiated the project this year, with a new strategy of deleting the entire gene (about 20 KB) by simultaneously injecting gRNAs targeting the length of the gene along with Cas9 in order to assure complete disruption of gene function. The strategy worked, leading to recovery of a founder line that appears to indeed lack the entire rca2.1 locus. We are currently assisting the Kemper lab with genetic and phenotypic characterization of the new allele.

ROLE OF MINERALS IN BONE HEALTH (LAVERNE BROWN, OFFICE OF DIETARY SUPPLEMENTS)
The NICHD Zebrafish Core welcomed LaVerne Brown as a Special Volunteer this year. Together, we devised a nutritional study that will be used to define fish feed from the Nutrition Obesity Research Center of the University of Alabama (Birmingham) in order to explore how certain minerals influence bone health in the presence or absence of adequate vitamin D, a topic of relevance to human bone health, as well as differences in the role of vitamin D between African Americans and other demographic groups.

Basic gene knockouts
The Core continues to offer the creation of at least two novel CRISPR/Cas9 frame-shifting alleles per gene on a fee-for-service basis. There were no basic gene knockout requests this year, as our customer laboratories are still characterizing mutant lines that we previously made for them. Since 2018, we have also been exploring the simultaneous targeting of multiple sites along a gene with CRISPR/Cas9 to create larger deletions, with a 20 KB deletion in rca2.1 (see Kemper lab above) as our pilot example. We were successful in finding a founder capable of germ-line transmission that follows clear Mendelian patterns of inheritance. Several other founders showed non-Mendelian patterns of inheritance, which we hypothesize are the result of CRISPR/Cas9–induced chromosomal structural alteration, leading to non-disjunction during meiosis. We plan to explore this possibility through whole-genome sequencing. The research should reveal general strategies and caveats in creating large deletion alleles so as to avoid the risk of false-negative results (i.e., reduction of phenotype strength), which are sometimes associated with standard CRISPR/Cas9 in-del alleles.

Independent research by the NICHD Zebrafish Core
OPTIMIZING STRATEGIES FOR CRISPR/CAS9–BASED HOMOLOGY-DIRECTED REPAIR (HDR)
Over the previous two years, the Core used CRISPR-Cas9 technology in combination with donor DNA to generate a zebrafish line with an atp7a amino acid (AA) substitution that is cognate to an ATP7A (AA) substitution of interest to the former Kaler lab (NICHD), a substitution that causes distal motoneuropathy. The
phenotype is characterized by hypopigmentation. We devised a novel screening strategy for this project that included synonymous changes alongside the targeted non-synonymous change. The synonymous changes introduced RFLPs (restriction fragment length polymorphisms) amenable to our molecular screening strategy. In collaboration with the NICHD Bioinformatics & Scientific Programming Core, software for generalizing this approach to any locus of interest has been developed, and we are currently preparing a manuscript summarizing these achievements. Over 2018–2019, the Core also conducted Directors Award–funded research in collaboration with the NICHD Molecular Genomics Core to compare precise genome editing methods and efficiencies using high-throughput sequencing. We compared five methods for generating seven alleles of interest to labs in NICHD and elsewhere at NIH, namely: two npc1 alleles (Porter lab, NICHD), one rhoaa allele (Weinstein lab, NICHD), one ifitm5 allele (Marini lab, NICHD), two ryr1b alleles (Lawal lab, NINR), and one cacna1c allele (Golden lab, NIDDK). We have extended the project for a third year to complete data analysis, prepare a manuscript on our findings, and recover successfully edited lines where we can. We are applying the same basic strategy, but with time-saving shortcuts, to a new project to create a precisely edited zebrafish satb1 mutant line with a nonsynonymous AA substitution that is cognate to a human disease-associated mutation of interest.

We continue to regularly meet and discuss progress with the laboratories of Raman Sood (NHGRI, Zebrafish Core) and Shawn Burgess (NHGRI) to ensure that efforts in optimizing precise genome editing strategies are not redundant.

**CRYO-PRESERVATION AND IN VITRO FERTILIZATION OF ZEBRAFISH SPERM**

Over the last year, we focused on improving quality control measures to ensure the viability of cryo-preserved zebrafish lines and minimize variability in viability. The studies continue with the additional goal of adding zebrafish-sperm health assessments to our repertoire of phenotype assessment. Our strategy involves pooling and aliquoting sperm from several carriers and conducing a test IVF from one aliquot.

**PREVENTING DNA DEGRADATION IN ZEBRAFISH BIOPSIES**

We have been working with the genotyping company Transnetyx to devise strategies for minimizing DNA degradation in zebrafish biopsies shipped to them for phenotyping. We have had good results with desiccation of biopsies prior to shipment and we are planning to write a manuscript describing the method.

**Institutional service**

**ACUC MEMBERSHIP**

Feldman has served on the NICHD Animal Care and Use Committee (ACUC) since 2015 and continued in this capacity this year, meeting monthly to evaluate and decide upon animal-study proposals, renewals and amendments, and ad hoc issues relevant to animal welfare.

**COVID–19 RESPONSE**

Feldman made a substantial contribution to coordinating and establishing standard operating procedures aimed at enabling scientists to safely continue and return to their zebrafish-related research.

**ZEBRAFISH SCIENCE FAIR DISPLAY**

Feldman worked closely with the Office of the Director to plan, design, and procure materials for a state-of-art display focusing on NIH Zebrafish research. The display was scheduled for the 2020 USA Science and Engineering Festival, which was canceled as a result of the Covid-19 pandemic. However, the steps taken prior
to the cancelation will greatly reduce the effort and costs required for completion and implementation of the display at a future event.

**Additional Funding**

- One-year salary support for Postbaccalaureate Fellow Emily Katz from the Office of Intramural Training & Education, NIH

**Publications**


**Collaborators**

- Perry Blackshear, PhD, *Signal Transduction Laboratory, NIEHS, Research Triangle Park, NC*
- LaVerne L. Brown, PhD, *Office of Dietary Supplements, Office of the Director, NIH, Rockville, MD*
- Steven Coon, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Ryan Dale, MS, PhD, *Bioinformatics and Scientific Programming Core; Computer Support Services Core, NICHD, Bethesda, MD*
- Andy Golden, PhD, *Laboratory of Biochemistry and Genetics, NIDDK, Bethesda, MD*
- Stephen Kaler, MD, *Section on Translational Neuroscience, NICHD, Bethesda, MD*
- Claudia Kemper, PhD, *Laboratory for Complement and Inflammation Research, NHLBI, Bethesda, MD*
- Tokunbor Lawal, PhD, *Neuromuscular Symptoms Unit, NINR, Bethesda, MD*
- Joan Marini, MD, PhD, *Section on Heritable Disorders of Bone and Extracellular Matrix, NICHD, Bethesda, MD*
- Forbes D. Porter, MD, PhD, *Section on Molecular Dysmorphology, NICHD, Bethesda, MD*
- Constantine Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*
- Brant Weinstein, PhD, *Section on Vertebrate Organogenesis, NICHD, Bethesda, MD*

**Contact**

For more information, email *bfeldman@mail.nih.gov* or visit *http://zcore.nichd.nih.gov*.
Affinity Groups

Scientists and physicians in the NICHD Division of Intramural Research (DIR) are organized into 12 affinity groups (AGs). Each AG is an intellectual hub for a group of investigators, creating a forum to share ideas and collaborate around common themes in support of the DIR mission.

The AGs serve as catalysts for new initiatives. Each investigator has a primary affiliation with an AG most closely aligned with his or her scientific interests. Secondary affiliations allow for communication across specialties in support of translational research and new collaborations.

Each AG has its own mission statement, shared research goals and objectives, and resources. Collectively, the AGs contribute to recruitment, mentoring, and the annual DIR scientific retreat.

Aquatic Models of Human Development
The Aquatic Models of Human Development Group uses zebrafish as a model vertebrate organism to study human development. We exploit the advantages of the zebrafish model for genetic screens, genomic manipulation, microscopic imaging, and cell and behavioral biology to gain insights into mechanisms controlling human development and defects therein that can result in disease. By maintaining a high level of expertise and resources, the group lowers the barrier to entry for other researchers in the intramural program for use of zebrafish as a research tool for investigating biological processes in vivo. The four PIs and the Zebrafish Core Facility, along with approximately 30 postdocs, doctoral trainees and technical staff, all focusing on zebrafish as a model system and all working in the Building 6 complex, create a critical mass of talent unsurpassed at NIH.

The group specializes in the generation of induced and engineered mutations and transgenic zebrafish lines that are used to study in vivo the function and integration of regulatory factors and pathways that are critical to the development, health, and survival of vertebrates, including humans. Extensive formal collaborations and informal interactions between group members promote the exchange of reagents and technical information to ensure that NICHD researchers remain at the cutting edge of zebrafish research worldwide.

Harold Burgess, Head
Ajay Chitnis
Katie Drerup
Ben Feldman (Core)
Brant Weinstein

Bone and Matrix Biology in Development and Disease
Matrix biology is central to the NICHD mission because it is intrinsic to the formation and reshaping of tissues before and after birth. This group aims to elucidate the mechanisms by which primary gene defects cause skeletal fragility and other matrix disorders and to apply this knowledge to the treatment of affected children. This goal is pursued with a close relationship between basic, translational, and clinical science.

The current primary focus of the group is on translational studies of skeletal dysplasias and related bone disorders. We have identified and characterized molecular mechanisms of several novel skeletal development disorders in this spectrum. In addition to building on these successes and expanding our knowledge of
mechanisms and pathology of skeletal development, we are developing novel approaches to therapeutic intervention, which will be translated to clinical trials. We are expanding our research to extracellular matrix (ECM) development and pathology in other tissues and organs. We also anticipate expanding our research program toward studies of ECM disorders in placenta, cartilage, growth plate, and other tissues and organs that are involved in fetal health, prematurity, and early child growth and development.

Joan Marini, Head
Sergey Leikin

Cell and Structural Biology
The Cell and Structural Biology Group (CSBG) conducts basic research on the molecular mechanisms that underlie fundamental cellular processes, such as organelle biogenesis and function, regulation of cell metabolism by small RNAs, small proteins, post-translational modifications and developmental programs, host-pathogen interactions, and the structure, traffic, and function of signaling proteins in the central nervous system. Knowledge gained from these studies is applied to the elucidation of the pathogenesis of various diseases, including neurodevelopmental, behavioral, and neurodegenerative disorders, metabolic disorders, and infectious diseases.

The CSBG has outstanding facilities for microscopic imaging of cell structure and function and for recombinant protein production in multiple prokaryotic and eukaryotic expression systems for X-ray crystallographic and biochemical analyses. In addition, the group has expertise in generating targeted mutations in rodents and in the anatomical, neurochemical, electrophysiological, and behavioral analyses of the mutant animals.

Matthias Machner, Head
Philip Adams
Anirban Banerjee
Juan Bonifacino
Andres Buonanno
Mary Lilly
Gisela Storz

Cell Regulation and Development
The mission and vision of the Cell Regulation and Development Group is to increase fundamental knowledge of basic molecular mechanisms of cell biology and animal development and enhance understanding of how dysregulation of these processes contributes to human disability and disease.

By combining expertise in the genetics of model organisms (including yeast, fruit fly, frog, zebrafish, and mouse), cell biology, biochemistry, molecular biology, electrophysiology, biophysics, genomics, and structural biology, members of the Group advance individual research objectives by regularly providing insights and advice to one another and through collaborations enabling synergy in research methods and experimental approaches. These interactions have engendered the development of novel technologies and strategies that underlie past accomplishments and will facilitate future discoveries by the Group in areas including the mechanisms and regulation of protein synthesis and transcriptional activation of gene expression, the functions and regulation of hormone receptors, signaling events and their responsive genes, mechanisms governing accurate segregation of the genetic information during cell division, mechanisms of nucleocytoplasmic trafficking, RNA processing
and RNA export, technologies based on transposable elements and deep sequencing for genome-wide profiles of gene function, the role of transposable elements in reorganizing the host genome in response to stress, technologies for analyzing gene expression at the single cell level during development, transcriptional control of the specification and differentiation of cells during vertebrate embryogenesis, the mechanisms governing adult organ formation during postembryonic vertebrate development, and molecular mechanisms of synaptic circuit assembly and function.

### Developmental Endocrinology, Metabolism, Genetics, and Endocrine Oncology

The mission of the **Developmental Endocrinology, Metabolism, Genetics and Endocrine Oncology (DEMGEO) Affinity Group** is to advance our understanding of endocrine, genetic, and metabolic disorders and endocrine tumors that impair human development, causing disease and disability. The research in this group encompasses basic, translational, and clinical science to elucidate the etiology of these diseases and to develop new diagnostic and therapeutic approaches.

<table>
<thead>
<tr>
<th>Karel Pacak, <em>Head</em></th>
<th>Deborah Merke (CC)</th>
<th>Stanko Stojilkovic</th>
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<tr>
<td>Jeff Baron</td>
<td>Anil Mukherjee</td>
<td>Constantine Stratakis</td>
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<td>Janice Chou</td>
<td>Forbes Porter</td>
<td>Jack Yanovski</td>
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<td>Fady Hannah-Shmouni</td>
<td>Kristina Rother (Training Program)</td>
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### Genetics and Epigenetics of Development

The mission of the **Genetics and Epigenetics of Development Group** is to research the genetic and epigenetic mechanisms that control reproduction, embryogenesis, and organ system development. Our vision is to apply the knowledge gained to enhance human health.

<table>
<thead>
<tr>
<th>Judy Kassis, <em>Head</em></th>
<th>Todd Macfarlan</th>
<th>Karl Pfeifer</th>
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<tr>
<td>Paul Love</td>
<td>Keiko Ozato</td>
<td>Pedro Rocha</td>
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### Genomics and Basic Mechanisms of Growth and Development

The mission of the **Genomics and Basic Mechanisms of Growth and Development (GBMGD) group** is to do basic research into the molecular mechanisms of fundamental processes ubiquitous to all cells. Such research inevitably results in new knowledge that impacts understanding of both health and disease. The members of the GBMGD group have a strong history of producing knowledge-changing advances in a number
of fundamental processes that are essential to life; DNA replication, DNA repair, nucleotide metabolism, RNA biogenesis and metabolism, chromatin-mediated control of gene expression, and genome integrity. Simply put, the GBMGD is a unique group of world experts in processes involving DNA and RNA metabolism and the consequences of alterations of these processes to cells and organisms. Recent advances from work by GBMGD members, as well as others, has revealed that although these processes are fundamental and ubiquitous, defects in them are often manifested as specific health disorders with distinctive phenotypes affecting human development and with surprising tissue-specificity, or in cancer. Indeed, a substantial number of the most common clinically relevant genetic disorders affecting children are due to polymorphisms/mutations in factors required for the maintenance of basal cellular functions. Inherent to the GBMGD mission is to increase understanding of how natural genetic diversity in the human population contributes to these fundamental processes in ways that affect health and disease, and to apply such knowledge so that specific strategies toward improving health can be developed.

The vision of the GBMGD group is to elucidate new knowledge about fundamental processes that will promote discovery of novel strategies for treatment and prevention alternatives across a multitude of diseases that share involvement of altered nucleic acid metabolism, gene expression and genome integrity. Through basic research that incorporates modern advances in biochemistry, genetics and genomics, GBMGD group members will continue to generate new knowledge relevant to the fundamental processes essential to growth, development and health.

The GBMGD group vision includes the promotion of collaborations and communication that support its mission. This group has the ability to discover fundamental aspects of growth and development from multifaceted perspectives and disciplines, and to investigate how disturbances in one process can affect another. Because our interests are not principally focused on any particular disorder or discipline, they extend beyond classical developmental biology and the gene pattern-specific expression aspects of animal development. By focusing on basal cellular functions, the GBMGD approach provides unique and complimentary perspectives on developmental biology and growth in health and disease states. Given the NIH’s tolerance to high-risk endeavors not usually fostered elsewhere, the GBMGD group, and the many collaborations among its members, fosters a greater depth and breadth of fundamental discovery than would exist in its absence.

Rich Maraia, Head
Mike Cashel
David Clark
Bob Crouch
Mel DePamphilis
Roger Woodgate

Maternal–Fetal Medicine and Translational Imaging

The mission of the Maternal–Fetal Medicine and Translational Imaging Affinity Group is to understand the biology of normal pregnancy and its complications, as well as normal/abnormal neurodevelopment during childhood by the application of innovative, state-of-the art cellular, molecular, and imaging methods to improve the diagnosis, treatment, and prevention of conditions responsible for maternal, perinatal, and infant morbidity and mortality.

Quantitative Imaging and Tissue Sciences (Basser) invents, develops, and translates novel in vivo microstructural
and functional MRI methods designed to measure salient properties of the developing brain and assess and characterize their changes in diseases and disorders. These novel quantitative imaging biomarkers are also used in neuroscience application to characterize brain network connectivity and dynamics, as well as brain tissue architectural organization.

**Nervous System Development and Plasticity (Fields):** Our research is concerned with understanding the molecular and cellular mechanisms by which neural activity and experience regulates development and plasticity of the nervous system, with three main areas of emphasis: (1) myelination and neuron-glia interactions, (2) cellular mechanisms of learning, and (3) gene regulation by neuronal firing.

**Translational Biophotonics (Gandjbakhche)** uses multi-disciplinary approaches to devise functional imaging technologies and methodologies for translating benchtop studies to the bedside. For example, near infrared spectroscopy and electroencephalogram are used to assess biomarkers for a wide range of brain development abnormalities and injuries, specifically, but not limited to, cognitive and behavioral disorders in children and traumatic brain injury. The laboratory explores endogenous (scattering and absorption) and exogenous (using fluorescence probes) optical contrast mechanisms for characterizing abnormal development and function in tissues such as the placenta. They also are involved in clinical and preclinical studies aimed at characterizing growth and development of various abnormal tissues and monitoring the efficacy of their treatment using photonics methods, such as fluorescence life time and multi spectral imaging.

**Intercellular Interactions (Margolis)** studies viral and non-viral pathogenesis in the context of human tissues. The laboratory developed a system of *ex vivo* human tissues that preserves their cytoarchitecture and important *in vivo* functions, and it studies lymphoid, cervico-vaginal, and placenta tissues to investigate mechanisms of cell-cell, cell-pathogen, and cell-extracellular vesicles interaction under normal as well as disease conditions.

**Perinatology Research (Romero)** investigates normal pregnancy and its most frequent complications such as preterm labor, preterm prelabor rupture of membranes, preeclampsia, fetal growth disorders, and fetal death—conditions which account for the excessive rate of infant mortality in the United States. The Laboratory conducts clinical and translational research and develops diagnostic, predictive, therapeutic, and preventative methods to reduce adverse pregnancy outcomes.

**Biomedical Optics (Tromberg)** develops models, methods, and devices for understanding and controlling light interactions with biological tissues. These methods are used to perform real-time quantitative measurements of clinically-relevant information, including: tissue blood flow, oxygen extraction, metabolic rate of oxygen consumption, and body/tissue composition (lean mass, hydration, and fat mass). Advanced capabilities include continuous dynamic monitoring of intrinsic physiological signals that can be used in feedback optimization for guiding therapies and clinical decision making. Our technology development effort includes portable, bedside, non-contact and wearable sensor platforms, as well as the design and integration of probes into instruments for minimally invasive surgical feedback and guidance.

Roberto Romero, *Head*  
Peter Basser  
R. Douglas Fields  
Amir Gandjbakhche  
Leonid Margolis  
Bruce Tromberg (NIBIB)
Molecular Medicine
The Molecular Medicine Group brings together basic research programs that share the ultimate goal of developing treatments for human diseases through a better understanding of their pathophysiology. Main areas of research focus include the maintenance of iron homeostasis and mechanisms underlying neurodegeneration. At the intersection of our work, we hope to shed light on a group of rare diseases called neurodegeneration with brain iron accumulation or NBIA, which may also have broader implications for other types of related diseases.

Claire Le Pichon, Head
Tracey Rouault

Neurosciences
Understanding the structure and function of the nervous system is a prerequisite for predicting and treating neuropathologies. Our group uses a variety of preparations, including animal models and human tissue, and a variety of techniques to study the biology of development and function of the nervous system and underlying basic biological processes in both health and disease.

Mark Stopfer, Head
Tamás Balla
Dax Hoffman
Y. Peng Loh
Chris McBain
Tim Petros

Physical Biology and Medicine
Human development, on which the future child’s health depends, is a complex phenomenon within the female starting with egg-spermatozoa fusion. In each individual, a plethora of molecular recognition events mediate the development of an immune system to defend against pathogens, a musculoskeletal system to maintain the body, and flexible networks of molecular expression to manage environmental stress. Traditionally, studies of these processes are divided into biochemistry, cell biology, virology, toxicology, etc. However, nature does not know these artificial divisions, and new understandings emerge from the crucible that interfaces mathematically minded physical scientists with biomedical researchers. The Physical Biology and Medicine Group is a unique scientific body that approaches human development in normal life and pathology as an integral process and encompasses first-class cell biologists, physical chemists, biophysicists, virologists, and immunologists who not only successfully train postdocs and students within their own fields, but widely collaborate, building and uniquely promulgating multidisciplinary approaches to the most important biomedical problems in the framework of the NICHD mission.

By choosing carefully which biophysical projects really answer the fundamental problems that limit advancement in medicine, we as a group will suffuse these identified problems with our basic wisdom of biophysics. This wisdom includes a deep knowledge of polymer physics, membrane biology and virology, inter- and intracellular communication, the theory of transporters and diffusion of domains, the physics of channel permeation and protein conformational change, the physical chemistry of membrane hydration forces and recognition, the physiology of cell signaling and receptor activation, lipid/protein interactions, and the physiology of secretion, viral infection, parasite invasion, fertilization, adipose transporter trafficking
and insulin signaling, and developmental cell fusion. Our strategy is to sharpen our techniques and power of observation to prove our discoveries, including proteomic architecture of signaling complexes and cellular structures, TIRF microscopy, long-term live-cell time-lapse imaging of tissue and cultured cells, confocal and two-photon scanning microscopy, electrophysiology, physical theories to devise experimental tests of hypotheses, cryoelectron microscopy, and lipidomic analyses.

We are now in a position to tackle the developmental changes seen in obesity and genetic disorders, the pathophysiology of influenza, dengue, and HIV viral infection and assembly, a 3D immunohistochemical microscopy of normal and compromised placenta at super-resolution levels, the use of human brain culture in dissociated and slice culture to study traumatic brain injury and glioblastomal neoplasia, membrane domain organization, regulation of mitochondrial metabolism by cytosolic proteins, and muscle molecular ultrastructure. We currently anticipate testing if hydrogen bond theory can explain hydration repulsion of DNA and membranes, developing a deeper understanding of mechanical, electrical, and chemical interactions in complex, multicomponent cell communities, achieving the conceptual integration of cutting-edge immunological information and assays for cell-cell communication in the onset of tissue pathology and discovering the role and mechanisms of microRNA vehicles in serum.

Leonid Chernomordik, Head
Sergey Bezrukov

Alexander Sodt
Joshua Zimmerberg

Reproductive Endocrinology and Infertility and Pediatric and Adolescent Gynecology

The Reproductive Endocrinology & Infertility and Pediatric & Adolescent Gynecology Group consists of the primary NICHD investigators who carry out research and clinical care in women’s health. Our mission is to conduct innovative translational research and provide direct patient care in reproductive endocrinology and gynecology. Consultative services are provided to all NIH institutes.

Alan DeCherney, Head (Training Program)   Veronica Gomez-Lobo (Training Program)
NEUROSCIENCES

Phosphoinositide Messengers in Cellular Signaling and Trafficking

Every biochemical process in a eukaryotic cell relies on a molecular information flow that leads from receptors, which inform the cell about its environment, all the way to the molecular effectors that determine the appropriate cellular response. Proper information transmission requires a high degree of organization, where the molecular players are organized into different cellular compartments so that the specificity of the cellular response can be properly maintained. Breakdown of this organization is the ultimate cause of all human diseases, even if the affected molecular pathways differ according to the type of disease, such as cancer, diabetes, or neurodegenerative diseases, just to name a few.

Research described in this report focused on the question of how cells organize their internal membranes to provide a structural framework on which molecular signaling complexes assemble to ensure proper information processing, cellular processes that are often targeted by cellular pathogens, such as viruses, to force the cells to produce the pathogen instead of performing the cell's normal functions. Better understanding of such processes can not only provide new strategies to fight various human diseases but also intercept the life cycle of cellular pathogens, thus offering an alternative to antimicrobial drugs.

Intracellular distribution of phosphatidylinositol in living mammalian cells

Phosphorylated inositol phospholipids (PPIn) are a class of phospholipids that are present in tiny amounts but have very important regulatory functions, as they organize protein signaling complexes on specific membrane compartments. They are produced by phosphoinositide kinases that can phosphorylate specifically one of three positions of the inositol ring of one of the major classes of phospholipids, phosphatidylinositol (PI). Although the cellular localization of most PPIn has been thoroughly studied and mapped, the cellular distribution of PI, their more abundant precursor, has not been determined. In this review period, we developed new molecular tools to gain information on PI distribution in intact live mammalian cells without disrupting their membrane integrity. Using structural information available for bacterial PI–specific phospholipase C enzymes (PI-PLCs), we selected the highly active enzyme from *Bacillus cereus* to use it as a platform for protein engineering. *Bacillus cereus* (Bc)
PI-PLC shows remarkable specificity for PI and does not display catalytic activity towards phosphorylated PPIn species or other phospholipids. Capitalizing on such features and to mark the intracellular distribution of PI, we generated mutant forms of the enzyme targeting residues within the conserved catalytic domain that would abolish enzymatic activity but maintain substrate coordination within the active site. Fusion of this mutant BcPI-PLC to the green fluorescent protein (GFP) and expression in mammalian cells allowed visualization of the distribution of the protein by confocal microscopy in living cells. We then designed BcPI-PLC constructs with minimal interfacial binding, and therefore low basal catalytic activity from the cytosol. Using the rapamycin-inducible heterodimerization system, we modified the enzymes, which were capable of rapidly hydrolyzing PI when recruited in the proximity of membrane-embedded substrate. Acute targeting of this recruitable BcPI-PLC to various organelles, together with detection of diacylglycerol (DAG), the product of PI hydrolysis, could serve as a proxy to assess the PI content of that membrane. Our findings using these tools showed that PI is localized to the endoplasmic reticulum (ER), the site of its synthesis, but is also enriched in the cytosolic leaflets of the Golgi complex, peroxisomes, and mitochondria. Strikingly, we did not find significant amounts of PI within the plasma membrane (PM) or in endosomal compartments in any of the mammalian cell types examined.

As part of these studies, we also developed bioluminescence energy-transfer (BRET)–based biosensors to monitor organelle-specific PI, DAG, and PPIn dynamics at the level of cell populations and combined the method with the use of the recruitable BcPI-PLC construct to characterize the role of PI availability and supply for the generation of PPIn species within distinct membrane compartments of live cells. The studies reveal the explicit need for the sustained delivery of PI from the ER, rather than its absolute steady-state content, to maintain monophosphorylated PPIn species within the PM, Golgi complex, and endosomal compartments. Overall, our findings mapped for the first time the PI distribution in mammalian cells and revealed a suspected, yet never formally proven, role for PI transfer and substrate channeling in the spatial control of PPIn metabolism. The importance of the studies is that, using these new molecular tools, researchers will be able to characterize the biochemical processes and their molecular machinery that are responsible for the transport of PI from the ER and how these molecules contribute to establish the proper lipid composition of organelle membranes.

**Role of the phosphatidylinositol 4-kinase beta–interacting c10orf76 protein in viral replication**

As mentioned in above, viral replication requires host factors that many viruses hijack to establish their optimal membrane niche for their replication. Targeting such processes would be an effective means of combating virus infections and propagation. In a separate set of studies, we collaborated with the groups of John Burke and Frank van Kuppeveld to understand the role of the enigmatic c10orf76 protein in viral replication with respect to its interaction with the lipid kinase phosphatidylinositol 4-kinase B (PI4KB). PI4KB is a lipid kinase that generates phosphatidylinositol 4-phosphate (PI4P), a critical regulatory lipid in the Golgi complex. PI4KB has been identified as one of the essential host factors necessary for replication of several small picornaviruses in mammalian cells. PI4KB can interact with many protein-binding partners, which are differentially manipulated by picornaviruses to facilitate replication. The protein c10orf76 is a PI4KB–associated protein that increases PI4P levels at the Golgi and is essential for the viral replication of specific enteroviruses. In this collaborative effort, the Burke lab used hydrogen-deuterium exchange mass spectrometry to characterize the c10orf76–PI4KB complex. The studies revealed that c10orf76 and PI4KB directly interact, that binding is mediated by the kinase linker region of PI4KB, and that formation of the heterodimeric complex is modulated by protein kinase A (PKA)–dependent phosphorylation. Using mutant proteins that interrupt the association between PI4KB and c10orf76, we found that PI4KB is required for the
recruitment of c10orf76 to the Golgi, but that PI4KB will still bind to the Golgi, without c10orf76 protein. van Kuppeveld’s group showed that, while all enteroviruses require PI4KB for replication, replication of c10orf76–dependent enteroviruses requires intact c10orf76–PI4KB interaction, whereas viruses whose replication is independent of c10orf76 still require PI4KB but can replicate with PI4KB mutants that are unable to recruit c10-rf76. The studies also revealed that c10orf76 controls the small GTP-binding protein Arf1 and that the regulation is important to maintain the level of PI4P in the Golgi complex. The studies’ significance is that they characterized important protein components of human cells that are used by some viruses for their replication, knowledge that will be used to assess the host protein requirement for the replication of any future viruses that pose a health risk to the public.

**Identification of a new membrane sub-compartment in secretory cells**

Our group was involved in a multicenter study that identified ribosome-associated vesicles as a dynamic sub-compartment of the ER in secretory cells. Using a combination of live-cell microscopy with *in situ* cryo-electron tomography, ER dynamics in several secretory cell types, including pancreatic β-cells and neurons, were directly visualized under near-native conditions, imaging approaches that identified a novel, mobile vesicular compartment of the ER, characterized by the presence of ribosomes. The ribosome-associated vesicles (RAVs) were found primarily at the cell periphery, observed across different cell types and species. The studies showed that RAVs exist as distinct, highly dynamic structures separate from the intact ER reticular architecture and that they also interact with mitochondria via direct intermembrane contacts. The findings described a new ER sub-compartment within cells, the function of which is still under investigation.

**Additional Funding**

- Natural Sciences & Engineering Research Council of Canada (NSERC) Banting Postdoctoral Fellowship supporting Dr. Joshua Pemberton

**Publications**

Collaborators

• Evžen Boura, PhD, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic
• John Burke, PhD, University of Victoria, Victoria, Canada
• Zachary Freyberg, MD, PhD, University of Pittsburgh, Pittsburgh, PA
• Juan Marugan, PhD, Division of Pre-Clinical Innovation, NCATS, Bethesda, MD
• Radim Nencka, PhD, Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic
• Mark Stopfer, PhD, Section on Sensory Coding and Neural Ensembles, NICHD, Bethesda, MD
• Frank van Kuppeveld, PhD, Utrecht University, Utrecht, The Netherlands.
• Péter Várnai, MD, PhD, Semmelweis University, Faculty of Medicine, Budapest, Hungary

Contact

For more information, email ballat@mail.nih.gov or visit http://ballalab.nichd.nih.gov.
Molecular mechanism of post-translational protein lipidation by DHHC palmitoyltransferases

Post-translational modifications greatly expand the structural, chemical, and functional diversity of the proteome. Of these, protein lipidation, which collectively refers to covalent modification of proteins by lipids, constitutes a centrally important class of post-translational modification. Protein palmitoylation, or more generally, protein S-acylation is a specific form of protein lipidation, whereby long-chain, typically C16, fatty acids become covalently attached to internal cysteines through a thioester linkage. Palmitoylation is one of the most pervasive and physiologically important post-translational modifications, and the targets of palmitoylation span a very wide range of proteins, ranging from ion channels to cell-surface receptors, neuronal scaffolding proteins, and small GTPases. The repertoire of palmitoylated proteins has expanded rapidly in recent years, with thousands of proteins now known to be part of the cellular “palmitoylome.” The physicochemical effect of palmitoylation is to alter the local hydrophobicity of the substrate protein. The thioester bond makes S-acylation unique in that it is a labile moiety and can be cleaved, in the cellular context, by thioesterase enzymes, which makes S-acylation one of the few dynamic post-translational modifications and unique among different forms of protein lipidation. The physiological effects of S-acylation are diverse and have critical cellular importance; for example, Ras, a small GTPase that is critical for cellular growth and differentiation and is mutated in about one-third of all human cancers, is palmitoylated at the Golgi and subsequently targeted to the plasma membrane by vesicular transport. Palmitoylated Ras localizes to cholesterol-rich domains on the plasma membrane. However, it is subsequently depalmitoylated by the thioesterase APT1, dissociates from the plasma membrane, and redistributes on endomembranes, including the Golgi. Such dynamic recycling of Ras is critical for its function.

Protein S-acylation is catalyzed by a large group of enzymes known as DHHC-palmitoyl acyltransferases (DHHC-PAT), so named because they contain a signature D-H-H-C motif (aspartate-histidine-histidine-cysteine) in a cysteine-rich domain (CRD) in an intracellular loop (Figure 1). These are low-abundance polytopic integral membrane
proteins localized at a variety of cellular compartments. Humans have 23 DHHC-PATs encoded in their genome. Beyond the shared DHHC domain, DHHC-PATs vary considerably; some possess ankyrin repeats (structural protein motifs that mediate protein-protein interactions), a few have six transmembrane helices instead of the usual four, and at least one forms a functional heterodimer with a cytoplasmic auxiliary subunit (Figure 1). To date, no consensus sequences have been reported for palmitoylation. A specific DHHC-PAT can palmitoylate many substrates, and, conversely, a given substrate can be palmitoylated by many DHHC-PATs. Such redundancy has been one of the most intriguing aspects of DHHC-PATs and makes it difficult to assign substrates by overexpression/knockout strategies, given that, in the absence of one DHHC-PAT enzyme, others can take over. However, this does not necessarily reflect the true enzyme-substrate relationship. The situation has been even more confounded by the lack of specific inhibitors of DHHC-PATs. Even though 2-bromopalmitate is widely used as a global inhibitor of DHHC-PATs, it has been shown that it also broadly targets other proteins involved in lipid metabolism.

Besides its broad importance in cell biology, palmitoylation has been linked to several diseases, most notably neuropsychiatric disorders such as Huntington's disease and various forms of cancer. Recently, it was shown that DHHC20 palmitoylates epidermal growth factor (EGFR) and is thus a potential therapeutic target for a wide range of cancers. More recently, DHHC3 has been proposed as a target for cancer treatment owing to its activity as the palmitoyltransferase for programmed cell-death ligand 1 (PD-L1). However, when we started working on this family, very little was known about the molecular mechanism of DHHC palmitoyltransferases, despite their importance across a broad spectrum of biological pathways and their biomedical importance. Nothing was known about their structural organization or how they interact with substrates and the fatty acyl coenzyme A (CoA), which serves as the acyl donor.

In a major breakthrough in this field, we had earlier solved the high-resolution crystal structures of two members of the DHHC family, human DHHC20 (hDHHC20) and zebrafish DHHC15 (Figure 2a). They are the
a. The structure of human DHHC20 and a catalytically inactive mutant of zebrafish DHHC15, shown in ribbon trace. The transmembrane domain (TM) is shown in green, the DHHC-containing cysteine-rich domain in blue and the C-terminal domain in orange. The grey spheres indicate Zn$^{2+}$ ions. These are both Golgi-resident enzymes, and thus the top side faces the Golgi lumen and the active site the cytoplasm.

b. Active site of human DHHC20 showing the catalytic triad containing the active-site cysteine.

c. Structure of human DHHC20 irreversibly modified with 2-bromopalmitate, which results in the active-site cysteine linking to the alpha-carbon of palmitic acid. The acyl group of palmitic acid is shown in stick rendition. Also shown are two residues towards the top of the tapering cavity to where the palmitate binds.

d. The acyl chain-length selectivity of wild-type (WT) human DHHC20. Mutation of tyrosine181 to alanine (Y181A) expands the cavity and shifts the acyl selectivity to the longer side. On the other hand, mutation of serine29 to phenylalanine (S29F) contracts the cavity and thus shifts the acyl selectivity to the shorter side.

e. 3D density maps for the POPC alkyl-chain core (yellow surface) near the protein surface (blue), calculated from the molecular dynamics (MD) trajectories. The catalytic cysteine (Cys156) is highlighted (magenta). The four regions where the membrane is deformed are indicated. The approximate minimum and maximum widths of the alkyl-chain core of the bilayer are also indicated, showing that the maximum deformation is around the active site.

First structures of any member of this family to be characterized and reveal a tepee-like transmembrane domain organization, which splays apart towards the cytoplasmic side and harbors the active site at the membrane-aqueous interfacial region (Figure 2b), thus readily explaining why membrane-proximal cysteines are palmitoylated. We also solved the structure of hDHHC20 irreversibly modified by a covalent inhibitor, 2-bromopalmitate. The structure mimics the auto-acylated intermediate state in the enzymatic pathway and thus reveals how the acyl group of fatty acyl–CoA binds in a cavity formed in the bilayer by the transmembrane domain (Figure 2c). Residues lining the cavity contact the acyl chain, and their mutation affects enzymatic activity. By mutating two residues at the tapering end of the cavity, we also showed that we can change the acyl chain-length selectivity of the mutant enzymes (Figure 2d). Thus, the cavity functions as a molecular ruler in determining the acyl chain-length selectivity of DHHC20, important because, although palmitate is the most prevalent fatty acid used by DHHC palmitoyltransferases, they can use fatty acyl–CoAs of other chain lengths, a property that varies between different members of the DHHC family.
In the past year, we focused on structure-based understanding of several aspects of DHHC function. Starting from our high-resolution structure and using molecular dynamics simulations and in collaboration with José Faraldo-Gómez, we showed that the protein surface of hDHHC20 severely distorts the membrane, which causes the active site to be exposed to the aqueous side of the membrane. Otherwise, the active site would have been buried in the hydrophobic part of the membrane and would have impaired catalysis, given that this kind of catalysis involves charge separation. Using a combination of X-ray crystallography and atomistic molecular dynamics simulations, we also determined a high-resolution structure of human DHHC20 in complex with an intact palmitoyl CoA. The structure represents the pre-catalytic complex of hDHHC20 with palmitoyl CoA and reveals how the polar CoA part of the fatty acyl-CoA is recognized by hDHHC20. Mutation of residues involved in binding to CoA impair catalysis, thus lending credence to our structural model. We also showed that recognition of palmitoyl CoA by hDHHC20 depends on the coincident recognition of both the fatty

**FIGURE 3. Iron transport by Mitoferrin and MavN**

*a.* Iron is imported through the plasma membrane by the Transferrin/Transferrin Receptor (*blue*) cycle and is transported out of endosomes by the divalent metal ion transporter (DMT) (*grey*). Iron is delivered to Mitoferrin (*yellow cylinders*) by unknown means. Mitoferrin delivers iron to unknown partners in mitochondria, which become available for heme and Fe-S cluster biosynthesis.

*b.* Schematic depiction of the simplest transporter cycle and the different putative states of Mitoferrin involved.

*c.* Assay of iron-transport activity by TMfrn1, a Mitoferrin1 homolog. Representative PGSK (fluorescent reporter of iron) quenching curves upon addition of iron to TMfrn1 proteoliposome. Red traces are increasing concentrations of iron. Black dotted traces show protein-free liposomes.

*d.* Schematic showing *Legionella* entering a host cell and sequestering itself in a *Legionella*-containing vacuole (LCV). MavN is inserted into the membrane of the LCV and hijacks iron from the host cell.

*e.* Assay showing iron transport by proteoliposome-reconstituted MavN, using the identical assay as in *c.*
acyl chain and the CoA headgroup, thus explaining how high concentrations of free intracellular CoA cannot inhibit DHHC enzymes.

**Molecular mechanism of Porcupine, an integral membrane enzyme of the MBOAT family, which catalyzes Wnt lipidation**

In metazoans, Wnt proteins regulate many processes, including cellular growth, differentiation, and tissue homeostasis, through the highly conserved Wnt signaling pathway. Porcupine is an endoplasmic reticulum (ER)-resident integral membrane enzyme, which catalyzes post-translational modification of Wnts with the unsaturated lipid palmitoleic acid. This unique form of lipidation with palmitoleic acid is a vital step in the biogenesis and secretion of Wnt. Porcupine inhibitors are currently in clinical trials for cancer treatment. However, Porcupine-mediated Wnt lipidation has not been reconstituted *in vitro* with purified enzyme. We recently reported the first successful purification of human Porcupine and confirmed, through *in vitro* reconstitution with the purified enzyme, that Porcupine is necessary and sufficient for Wnt acylation. By systematically examining a series of substrate variants, we showed that Porcupine intimately recognizes the local structure of Wnt around the site of acylation. Our *in vitro* assay enabled us to examine the activity of Porcupine with a range of fatty acyl–CoAs of varying length and unsaturation. The selectivity of human Porcupine across a spectrum of fatty acyl–CoAs suggested that the kink in the unsaturated acyl chain is a key determinant in Porcupine-mediated catalysis. We also showed that two putative Porcupine inhibitors, which were discovered with cell-based assays, indeed target human Porcupine. Together, the results provide several, high-resolution biochemical insights into the mechanism of Porcupine-mediated Wnt acylation and pave the way for further detailed biochemical and structural studies.

**Molecular mechanism of iron transport across cellular membranes**

The importance of iron in biology cannot be overstated. In higher organisms, mitochondria are the ‘hotspot’ for the cell biology of iron, because Fe-S clusters are biosynthesized and iron is inserted into heme there. Mitochondrial iron homeostasis plays a critical role in cellular iron homeostasis and in the overall physiology of the cell. In vertebrates, the only known major transporters of iron into mitochondria are mitoferrin-1 and mitoferrin-2, two homologous members of a large group of mitochondrial transporters known as the Mitochondrial Carrier family (Figures 3a and 3b). Mitoferrin-1 (Mfrn1) is expressed mainly in erythroid cells, while mitoferrin-2 is expressed ubiquitously. Knockout of Mfrn1 is embryonically lethal, reflecting the importance of mitoferrins in vertebrate physiology.

Mfrn1 and Mfrn2 were discovered more than 10 years ago. However, the proposed iron-transport activity had not been demonstrated using an *in vitro* functional reconstitution assay. Also no report about their interaction with iron or other related metal ions existed, most likely because heterologous overexpression and purification of mitoferrins were not reported in the literature. We carried out heterologous purification, *in vitro* functional reconstitution, and mutational dissection of a vertebrate Mfrn1 (Figure 3c), the first demonstration that Mfrn1 can indeed transport iron. We showed that Mfrn1 is a promiscuous metal ion transporter in that it also transports other first-row transition metal ions. Through mutagenesis, we discovered candidate residues that are important for metal-ion transport by Mfrn1 and those that could be involved in forming metal-ion binding sites during transport. Our studies provided the first biochemical insights into Mfrn function and form the starting point for future high-resolution structural studies of Mfrn function. Our transport assay and the purification strategy will lead to more detailed biochemical and biophysical experiments into the mechanistic basis of iron transport by Mfrn1 and Mfrn2.
In the past year, we used our in vitro proteoliposome-reconstituted iron-transport assay, the first such assay to be reported in the literature, to dissect the iron-transport activity of MavN, another proposed iron transporter, in the bacterial pathogen Legionella pneumophila. Legionella is an intracellular pathogen that enters the host cell and sequesters itself in the Legionella-Containing Vacuole (LCV), where it survives and proliferates. This is achieved by secreting hundreds of so-called “effector proteins,” which are secreted through the Type IV secretion system (T4SS) into the host cell. Of the hundreds of effector proteins, there are seven core effector proteins, and MavN is one of them. MavN is inserted into the membrane of the LCV. Using genetic and cell-based studies, the laboratory of our collaborator Ralph Isberg proposed that MavN is an iron transporter. By heterologous purification and proteoliposome reconstitution, we showed that MavN is indeed an iron transporter. Mutations in MavN that impaired iron transport in vitro also impaired Legionella growth in a cell-based assay, pointing to the importance of MavN for Legionella.

Structure and molecular mechanism of ATG9, the only transmembrane component of the core autophagy machinery

Autophagy is a critical process in both health and disease. Serving mainly to adapt organisms to a diverse range of conditions such as metabolic stress, pathogenic infection, and ageing, autophagy is conserved from yeast to humans. In addition, autophagy is involved in a large number of human diseases, including neurodegenerative disorders, various forms of cancer, and inflammatory diseases. Although autophagy was discovered more than 50 years ago, some of its fundamental aspects remain rather poorly understood and are only coming to light now. An outstanding example of this lack of knowledge is the structure and function of ATG9, the only essential transmembrane protein in the pathway. ATG9 localizes to small, 30–60 nm vesicles known as “ATG9 vesicles,” which are critically important for the expansion of the autophagosome membrane. ATG9 vesicles traffic between the trans-Golgi network (TGN) and pre-autophagosomal structures in response to stimuli that initiate autophagy. Despite the clear and obvious importance of ATG9 in autophagy, its atomic structure and molecular function remain unknown. It has been speculated that ATG9 transports lipids that contribute to the growth of the double-membrane structure of the autophagosome. However, progress in this regard has been completely thwarted by the lack of an atomic structure that could serve as a starting point for a range of experiments, from cell-biological to biochemical, to elucidate its function.

In order to address this knowledge gap, we initiated a collaboration with the labs of Juan Bonifacino and Jiansen Jiang. This led to a 2.9 Å–resolution structure of the ubiquitous human ATG9A isoform by cryo-EM (Figure 4a). The structure revealed that ATG9A adopts a unique fold (Figure 4b, 4c), assembling into a domain-swapped homotrimer, with each wedge-shaped protomer comprising four transmembrane α-helices and two α-helices that are only partially embedded in the membrane. The transmembrane domain is capped by a cytosolic domain, which is mostly α-helical. Classification of the cryo-EM imaging revealed two predominant ATG9A conformers (states A and B). An important feature of the ATG9A structure is the presence of a branched network of pores through the protein, with outlets facing the cytosol, the outer leaflet lipid head groups, and the lumen (Figure 4d). Cell-based functional assays demonstrated importance of pore-lining residues, suggesting an essential function of the pore. The dimensions of the pore are sufficient to accommodate phospholipids and likely permit lipid transport from or to a lipid chaperone such as ATG2. Alternatively ATG9 may act as a lipid scramblase, distributing the lipids that arrive through ATG2 among the two leaflets of the bilayer. The branched pores may also function as solvent conduits to relieve osmotic pressures in growing and shrinking ATG9 vesicles. ATG9A directly interacts with ATG2A, interactions that are mediated by the ATG9A cytosolic domain, deletion of which abrogates both ATG2A co-immunoprecipitation and autophagosome
maturation. Because ATG2A has demonstrated lipid-transport capability \textit{in vitro}, the plausible functions of ATG9A are to: (1) facilitate movement of lipids across the bilayer of the growing phagophore, which could be achieved by using the branched pores we see in our structure for moving polar lipid headgroups or by distorting the bilayer, lowering the energetic barrier for trans-bilayer lipid movement; (2) act as a membrane-anchored “lasso” to capture and accurately target ATG2A or other lipid chaperones. It is interesting to note that two helices, which plug the central pore of the trimer, are discernible only in state A, indicating that flexibility of the putative “lasso” could be partly dependent on the conformation of the transmembrane core.

The early stages of autophagy involve a reorganization of intracellular membranes to form the nascent autophagosome. ATG9 is essential for this process, and our structure provides a framework for interrogating its function in bilayer remodeling. Using molecular dynamics simulations in collaboration with the lab of José
Faraldo-Gómez, and based on the cryo-EM data, we found that the architecture of the ATG9A trimer elicits long-range positive membrane curvature, owing to the geometric features and amino-acid make-up of the protein surface exposed to the lipid. The simulations also show that co-localization of multiple ATG9A trimers greatly amplifies this effect and induces macroscopic changes in membrane morphology (Figure 4e, 4f). The finding corresponds with observations that ATG9 is present in small (30–60 nm) vesicles and thin tubules, and, at least in yeast, localizes to the edges of the growing phagophore. Interestingly, states A and B differ in the tilt-angle of the protomers relative to the membrane normal, implying a tolerance for a range of bilayer curvatures. This leads to the intriguing possibility that ATG9A-induced curvature may modulate the energetic scale and effect lipid diffusion out of the vesicles into another membrane.

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Publications

Collaborators
- Juan Bonifacino, PhD, Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD
- José Faraldo-Gómez, PhD, Theoretical Molecular Biophysics Section, NHLBI, Bethesda, MD
- Rodolfo Ghirlando, PhD, Laboratory of Molecular Biology, NIDDK, Bethesda, MD
- James Inglese, PhD, Assay Development & Screening Technology Laboratory, NCATS, Bethesda, MD
- Ralph Isberg, PhD, Tufts University School of Medicine, Boston, MA
- Jiansen Jiang, PhD, Laboratory of Membrane Proteins and Structural Biology, NHLBI, Bethesda, MD

Contact
For more information, email anirban.banerjee@nih.gov or visit http://banerjee.nichd.nih.gov.
Regulation of Childhood Growth

Children grow taller because their bones grow longer. Bone elongation occurs at the growth plate, a thin layer of cartilage found near the ends of juvenile bones. In the growth plates, new cartilage is produced through chondrocyte proliferation, hypertrophy, and cartilage matrix synthesis, and then the newly formed cartilage is remodeled into bone. The process, termed endochondral ossification, results in bone elongation, which causes children to grow in height (linear growth). Consequently, mutations in genes that regulate growth-plate chondrogenesis cause abnormal bone growth and short stature in children. Depending on the severity and nature of the genetic abnormality, the phenotype can range from chondrodysplasias with short, malformed bones, to severe, often disproportionate, short stature, to mild proportionate short stature. If the genetic defect affects tissues other than the growth plate cartilage, the child may present with a more complex syndrome that includes other clinical abnormalities.

We investigate the cellular and molecular mechanisms governing childhood growth and development. We focus on growth at the growth plate, which drives bone elongation and therefore determines height. One goal of this work is to gain insight into the many human genetic disorders that cause childhood growth failure or overgrowth. A second goal is to develop new treatments for children with severe growth disorders.

Novel genetic causes of childhood growth disorders

For many children who are brought to medical attention for linear growth disorders, clinical, laboratory, and genetic evaluation fails to identify the underlying etiology. Genome-wide association studies and molecular studies on growth-plate biology suggest that there are hundreds of genes that control linear growth. Therefore, it is likely that many genetic causes of linear growth disorders remain to be discovered.

To discover new genetic causes of childhood growth disorders, we invite families with monogenic growth disorders to the NIH Clinical Center, where we evaluate the clinical, biochemical, and radiological
features of the condition. We then obtain DNA samples from informative family members and use powerful genetic approaches, including SNP arrays, to detect deletions, duplications, mosaicism, and uniparental disomy, combined with exome sequencing to detect single-nucleotide variants and small insertions/deletions in coding regions and splice sites. When sequence variants that are likely to cause the disorder are identified, we study the variants and the genes in which they occur in the laboratory to confirm that the variant is pathogenic, to elucidate the pathogenesis of the disorder, and to explore the role of the gene in normal growth.

We have used this approach to identify new causes of childhood growth disorders. For example, we previously found that variants in aggrecan (ACAN), a component of cartilage extracellular matrix, cause autosomal-dominant short stature with advanced skeletal maturation and that such patients also tend to develop early-onset osteoarthritis. We also found evidence that heterozygous deletion of CYP26A1 and CYP26C1, which encode enzymes that metabolize retinoic acid (RA), cause elevated RA concentrations, which accelerate bone and dental maturation in humans and cause developmental defects involving the eye and central nervous system. In additional studies, we found that variants in QRICH1, a gene of unknown function, cause a chondrodysplasia resulting from impaired growth-plate chondrocyte hypertrophic differentiation, in addition to the previously described developmental delay [Reference 1].

Recently, we applied this approach to evaluate a child with markedly delayed puberty, which was inherited as an autosomal dominant trait (Figure 1). Exome sequencing identified a rare missense variant (F900V) in DLG2, which co-segregated precisely with the delayed puberty phenotype in the extended family. DLG2 encodes PSD-93, an anchoring protein of N-methyl-D-aspartate (NMDA) receptors. Interestingly, NMDA receptors have been shown to control the timing of puberty in laboratory animals. We studied the impact of the identified variant in a GnRH neuronal cell line. GnRH neurons play a central role in the initiation of puberty. We found evidence that the variant impairs binding of PSD-93 to FYN (a protein tyrosine kinase), which diminishes phosphorylation and activation of NMDA receptors, leading to reduced GnRH expression (Figure 2). Next, we identified sequence variants in DLG2/PSD-93 in three subjects with isolated hypogonadotropic hypogonadism, a condition that leads to a failure of pubertal development. The variants also diminished binding to FYN and
reduced Gnrh1 expression in vitro. The findings provide evidence that variants in DLG2 cause delayed puberty and contribute to isolated hypogonadotropic hypogonadism by a mechanism that involves impaired binding to Fyn, diminished NMDA receptor phosphorylation and signaling, and consequently decreased GnRH expression [Reference 2].

Molecular and cellular mechanisms by which specific genes and pathways regulate childhood growth

Our group also studies the fundamental mechanisms governing skeletal growth. For example, we previously studied the role of EZH2 in the growth plate. EZH2 encodes a histone methyltransferase that catalyzes the trimethylation of histone H3 at lysine 27 (H3K27), which serves as an epigenetic signal for chromatin condensation and transcriptional repression. We found that loss of EZH1 and EZH2 in mice impaired bone growth, and we explored the cellular and molecular mechanisms involved. We also previously investigated the mechanisms that cause bones at different anatomical locations to vary dramatically in size and found that the postnatal developmental program that normally causes bone growth to decelerate with age plays out more rapidly in small bones than in large bones.

Recently, we studied the role of midkine in human growth, development, and disease. Midkine is a heparin-binding growth factor, which is highly expressed in several organs during embryogenesis. We previously found that it is present in high concentrations in human amniotic fluid and also in biopsy specimens of specific malignancies. We next analyzed plasma midkine concentrations in healthy, normal-weight children, healthy adults, obese children, girls and young women with anorexia nervosa, and children with idiopathic short stature. In healthy children, we found that plasma midkine concentrations declined with age. The decline occurred primarily during the first year of life. Plasma midkine did not significantly differ between males.
and females or between race/ethnic groups. Midkine concentrations were not correlated with body mass index (BMI) standard deviation score (SDS), fat mass (kg), or percent total body fat assessed by DXA (dual-energy X-ray absorptiometry); we found no difference in midkine between children with anorexia nervosa, healthy weight, and obesity. For children with idiopathic short stature, midkine concentrations did not differ significantly from normal-height subjects. The findings provide useful reference data for studies of plasma midkine in children with malignancies and other pathological conditions [Reference 3].

New treatment approaches for growth plate disorders

Recombinant human growth hormone (GH) is commonly used to treat short stature in children. However, GH treatment has limited efficacy, particularly in severe, non-GH–deficient conditions such as chondrodysplasias, and has potential off-target effects. Systemic insulin-like growth factor 1 (IGF-1) treatment has similar deficiencies. There are several endocrine and paracrine factors that promote chondrogenesis at the growth plate, which could potentially be used to treat such disorders. Targeting the growth factors specifically to the growth plate might augment the therapeutic skeletal effect, while diminishing undesirable effects on non-target tissues. To develop growth plate–targeted therapy, we previously used yeast display to identify single-chain human antibody fragments that bind to cartilage with high affinity and specificity. As a first test of the approach, we created fusion proteins combining the cartilage-targeting antibody fragments with IGF-1, an endocrine/paracrine factor that positively regulates chondrogenesis. Such fusion proteins retained both cartilage binding and IGF-1 biological activity, and they were able to stimulate bone growth in an organ culture system. Using a GH–deficient mouse model, we found that subcutaneous injections of the fusion proteins increased growth plate height without increasing proliferation in kidney cortical cells, demonstrating greater on-target efficacy at the growth plate and less off-target effect on the kidney than does IGF-1 alone. Our findings provide proof of principle that targeting therapeutics to growth plate cartilage could improve treatment for childhood growth disorders [Reference 4].

We are currently applying the approach to target other endocrine and paracrine factors that stimulate chondrogenesis to the growth plate. We are exploring the utility of the approach to both stimulate growth-plate chondrogenesis non-specifically and also reverse specific genetic defects in growth-plate function by modulating the abnormal molecular pathway responsible for the growth failure.

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Publications


**Collaborators**

- Greti Aguilera, MD, *Scientist Emeritus, NICHD, Bethesda, MD*
- Angela Delaney Freedman, MD, *St. Jude Children’s Research Hospital, Memphis, TN*
- Lijin Dong, PhD, *Genetic Engineering Core, NEI, Bethesda, MD*
- Ellen Leschek, MD, *Division of Diabetes, Endocrinology, and Metabolic Diseases, NIDDK, Bethesda, MD*
- Thomas Markello, MD, PhD, *undiagnosed Diseases Program, NHGRI, Bethesda, MD*
- Madhusmita Misra, MD, *Harvard Medical School, Massachusetts General Hospital, Boston, MA*
- Ola Nilsson, MD, PhD, *Karolinska Institute, Stockholm, Sweden*
- Sally Radovick, MD, *Rutgers Biomedical and Health Sciences, Robert Wood Johnson Medical School, New Brunswick, NJ*
- Katherine W. Roche, PhD, *Receptor Biology Section, NINDS, Bethesda, MD*
- Jack Yanovski, MD, PhD, *Section on Growth and Obesity, NICHD, Bethesda, MD*

**Contact**

For more information, email [jeffrey.baron@nih.gov](mailto:jeffrey.baron@nih.gov) or visit [http://baron.nichd.nih.gov](http://baron.nichd.nih.gov).
Quantitative Imaging and Tissue Sciences

In our tissue sciences research, we strive to understand fundamental relationships between function and structure in living tissues. Specifically, we are interested in how tissue microstructure, hierarchical organization, composition, and material properties affect their biological function or dysfunction. We investigate biological and physical model systems at various length and time scales, performing biophysical measurements in tandem with developing physical/mathematical models to explain their functional properties and behavior. Experimentally, we use water to probe tissue structure and function from nanometers to centimeters and from microseconds to lifetimes. We employ atomic force microscopy (AFM), small-angle X-ray scattering (SAXS), small-angle neutron scattering (SANS), static light scattering (SLS), dynamic light scattering (DLS), osmometry, and multi-dimensional nuclear magnetic resonance (NMR) relaxometry and diffusometry. A goal of our basic tissue-sciences research is to develop understanding and tools that can be translated from bench-based quantitative methodologies to the bedside.

Our tissue sciences activities dovetail with our basic and applied research in quantitative imaging, which is intended to generate measurements and maps of intrinsic physical quantities, including diffusivities, relaxivities, exchange rates, etc., rather than the qualitative images conventionally used in radiology. At a basic level, our work is directed toward making key invisible biological structures and processes visible. Our quantitative imaging group uses knowledge of physics, engineering, applied mathematics, imaging and computer sciences, as well as insights gleaned from our tissue-sciences research to discover and develop novel quantitative imaging biomarkers that can detect changes in tissue composition, microstructure, or microdynamics with high sensitivity and specificity. The ultimate translational goal is to assess normal and abnormal developmental trajectories, diagnose childhood diseases and disorders, and characterize degeneration and trauma (such as TBI). Primarily, we use MRI as our imaging modality of choice because it is so well suited to many applications critical to the NICHD mission; it is noninvasive, nonionizing, requires (in most cases) no exogenous contrast agents or dyes, and is generally deemed safe and effective for use with mothers, fetuses, and children in both clinical and research settings.
One of our technical translational goals has been to transform clinical MRI scanners into scientific instruments capable of producing reproducible, highly accurate, and precise imaging data with which to measure and map useful imaging biomarkers for various clinical applications, including single scans, longitudinal and multi-site studies, personalized medicine, and genotype/phenotype correlation studies, as well as for populating imaging databases with high-quality normative data. From a more basic perspective, another goal has been to apply our various MRI tools and methodologies to advance the field of neuroscience, providing new means and methods to explore brain structure/function relationships.

**In vivo MRI histology**

The most mature *in vivo* MRI histological technology that we invented, developed, and clinically translated is Diffusion Tensor MRI (DTI), by which we measure and map $D$, a diffusion tensor of water within an imaging volume. Information derived from this quantity includes white-matter fiber-tract orientation, the orientationally averaged mean apparent diffusion constant (mADC), and other intrinsic scalar (invariant) quantities. Such imaging parameters have been used by radiologists and neuroscientists as non-invasive quantitative histological ‘stains.’ These MRI images are obtained by probing endogenous tissue water *in vivo* without requiring any exogenous contrast agents or dyes. The mADC is used to identify ischemic areas in the brain during acute stroke and to follow cancer patients’ responses to therapy. Our measures of diffusion anisotropy (e.g., the fractional anisotropy or FA) are also widely used to follow changes in normally and abnormally developing white matter and in many other applications, such as brain white-matter visualization. Our group also pioneered the use of fiber direction–encoded color (DEC) maps to display the orientation of the main association, projection, and commissural white matter pathways in the brain. To assess anatomical connectivity among various cortical and deep-brain gray-matter areas, we also developed DTI “Streamline” Tractography, which is used to brain track white-matter fibers to help establish “anatomical connectivity” and by neuroradiologists and neurosurgeons to plan brain surgeries so that they can spare ‘eloquent’ areas of the brain. Collectively, these advances helped inspire several large federally funded research initiatives, including the NIH Human Connectome Project (HCP) and the NIH Brain Initiative.

More recently, we invented and developed a family of advanced *in vivo* diffusion MRI methods to measure fine-scale microstructural features of axons and fascicles, which otherwise could only be assessed using laborious *ex vivo* histological methods (i.e., “microstructure imaging”). We have been developing efficient means for performing “k- and q-space MRI” in the living human brain, such as “Mean Apparent Propagator” (MAP) MRI, an approach that can detect subtle microstructural and architectural features in both gray and white matter at micron-scale resolution, several orders of magnitude smaller than the typical MRI voxel size. MAP-MRI also subsumes DTI, as well as providing a bevy of new *in vivo* quantitative ‘stains’ or biomarkers to measure and map. We also developed a family of diffusion MRI methods to ‘drill down into the voxel’ to measure features such as average axon diameter (AAD) and axon-diameter distribution (ADD) within and along large white-matter pathways, dubbing them CHARMED and AxCaliber MRI, respectively. After careful validation studies, we reported the first *in vivo* measurement of ADDs within the rodent corpus callosum. The ADD is functionally important, given that axon diameter is a critical determinant of axon or nerve conduction velocity and therefore the rate at which information flows along axon...
FIGURE 1. Diffusion Exchange Spectroscopy (DEXSY) reveals water exchange dynamics at sub-cellular length scales and sub-millisecond time scales.

The figure shows the exchange of water molecules between and among distinct water pools in neural tissue at sub-cellular length scales and sub-millisecond time scales. We developed means to perform DEXSY experiments at finer length and time scales than previously thought possible and are relevant to many cellular and subcellular processes. Peaks along the diagonal lines show water populations in different compartments, but as \( t_m \), the “mixing time,” increases, some water molecules migrate into different compartments, where their diffusivity may be different. The process is observable as the buildup of off-diagonal peaks. The rate at which water exchanges between the different water compartments or pools can be determined by studying the various water fractions and their evolution with respect to mixing time.

bundled, and helps determine the latencies or time delays between and among different brain areas. We then developed a companion mathematical theory to explain the observed ADDs in different fascicles, suggesting that they represent a trade-off between maximizing information flow and minimizing metabolic demands. We also developed novel multiple pulsed-field gradient (mPFG) methods and demonstrated their feasibility \textit{in vivo} on conventional clinical MRI scanners as a further means to extract quantitative features in the central nervous system (CNS) such as the AAD and other features of cell size and shape.

Although gray matter appears featureless in DTI brain maps, its microstructure and architecture are rich and varied throughout the brain, not only along the brain’s cortical surface, but also within and among its various cortical layers and within deep gray-matter regions. To target this tissue, we have been developing several noninvasive, \textit{in vivo} methods to measure unique features of cortical gray-matter microstructure and architecture that are visible in electron microscopy (EM) applications but currently invisible in conventional MRI. One example is diffusion tensor distribution (DTD) MRI. One of our long-term goals is to ‘parcellate’ or segment the cerebral cortex \textit{in vivo} into its approximately 500 distinct cyto-architectonic areas using noninvasive imaging methods. To this end, we are developing advanced MRI sequences to probe correlations among microscopic displacements of water molecules in the cortex as well as sophisticated mathematical models to infer distinguishing microstructural and morphological features of gray matter. We also pioneered several
promising multi-dimensional MRI relaxometry and diffusometry-based methods to study water mobility and exchange in gray and white matter. We believe that these will eventually be translated to the clinic to help identify changes in normal and abnormal development, as well as inflammation and trauma.

**Quantitative MRI biomarkers for pediatric applications**

MRI is considered safer than X-ray-based methods, such as computed tomography (CT), for scanning fetuses, infants, and children. However, clinical MRI still lacks the quantitative character of CT. However, the scope of conventional MRI applications is limited to revealing either gross morphological features or focal abnormalities, which result in regional differences in signal intensities within a given tissue. Clinical MRI also often lacks biological specificity necessary for developing robust and reliable imaging “biomarkers.” In particular, MRI assessment of normal brain development and developmental disorders has benefited greatly from the introduction of “quantitative” clinical MRI techniques, with which one obtains maps of meaningful intrinsic physical quantities or chemical variables that can be measured in physical units and compared among different tissue regions, in individual subjects, and within longitudinal and cross-sectional studies. Quantitative MRI methods, such as DTI, also increase sensitivity, providing a basis for monitoring subtle changes that occur, e.g., during the progression or remission of disease, by comparing measurements in a
single subject with normative values obtained from a healthy population. Quantitative MRI methods should continue to advance “precision medical imaging” studies, in which MRI phenotypic and genotypic data can be meaningfully melded and used for improved diagnosis and prognosis assessments.

To advance our quantitative imaging activities, we developed algorithms that generate a continuous, smooth approximation to the discrete, noisy, measured DTI field data so as to reduce noise and allow us to implement Streamline Tractography more reliably. We proposed a novel Gaussian distribution for the tensor-valued random variables that we use to design optimal DTI experiments and interpret their results. In tandem, we developed non-parametric empirical (e.g., Bootstrap) methods to determine the statistical distribution of DTI–derived quantities in order to study, e.g., the inherent variability and reliability of computed white-matter trajectories. Such parametric and non-parametric statistical methods enabled us to apply powerful hypothesis tests to assess the statistical significance of findings in a wide range of important biological and clinical applications that were tested using ad hoc statistical methods. We are also developing novel methods to register different brain volumes and to generate group-average DTI data or atlases from various subject populations based on the Kullback-Leibler divergence. However, much work remains to be done in order to address and remedy MRI artifacts so as to permit one to draw statistically significant and unbiased inferences from clinical DTI data obtained in longitudinal and multi-center studies, and particularly in single-subject studies.

We carried out key clinical studies that utilize novel quantitative MRI acquisition and analysis methods and whose aim is to improve accuracy and reproducibility in diagnosis and to detect and follow normal and abnormal development. One early example is the NIH Study of Normal Brain Development, jointly sponsored by the NICHD, NIMH, NINDS, and NIDA. This multi-center consortium, initiated in 1998, was intended to

FIGURE 3. Various quantitative microstructural “stains” obtained from Mean Apparent Propagator (MAP) MRI from cohort of healthy human subjects

placement propagators measured in a cohort of 12 healthy volunteers: diffusion tensor imaging (DTI) parameters, mean diffusivity (MD), fractional anisotropy (FA), and direction encoded color (DEC) map; along with MAP microstructural parameters: return-to-origin probability (RTOP), return-to-axis probability (RTAP), return-to-plane probability (RTPP), propagator anisotropy (PA), Gaussian propagator anisotropy (PA_{mn}), non-Gaussian anisotropy (\Theta_{ng}), non-Gaussianity (NG), axial non-Gaussianity (NG_x), and radial non-Gaussianity (NG_\perp). These quantities highlight different microstructural features of gray and white matter and are obtained without any exogenous contrast agents or dyes, and without using ionizing radiation.
advance our understanding of normal brain development in typical healthy children and adolescents. The Brain Development Cooperative Group ([http://www.brain-child.org/brain_group.html](http://www.brain-child.org/brain_group.html)), created by this funding mechanism, is still actively publishing papers, primarily by mining the rich high-quality MRI data, many of which our lab processed, vetted, and uploaded, serving as the DTI Data-Processing Center (DPC) in this interdisciplinary project. The processed DTI data collected from the project were uploaded into a database accessible to interested investigators, and made publicly available through the National Database for Autism Research (NDAR; [http://ndar.nih.gov](http://ndar.nih.gov)). Carlo Pierpaoli, who spearheaded this work, continues to support, update, and disseminate the processing and analysis software called “TORTOISE,” that grew out of this effort and which can be downloaded from [http://www.tortoisedti.org](http://www.tortoisedti.org).

Traumatic Brain Injury (TBI) represents a significant public health challenge for our pediatric population, but also for young men and women in the military. Our involvement in TBI research, particularly in trying to detect mild TBI (mTBI), has continued to expand in partnership with various Department of Defense (DoD) affiliates. Diffusion MRI provides essential information to aid in the assessment of TBI, but it lacks sufficient specificity. Because of subtle changes seen in TBI, new sensitive and specific quantitative imaging protocols are required. To this end, we developed a DTI data–processing pipeline in order to improve the accuracy and reproducibility of MAP–MRI findings, and, in collaboration with scientists at the DoD Center for Neuroscience and Regenerative Medicine (CNRM), performed the first normative MAP–MRI studies, as well as applied this new and powerful method to detect tissue damage in brains of individuals who have suffered mild or moderate TBI. To enable this application, we are extending our NICHOD TORTOISE pipeline to analyze MAP–MRI data. We are now employing multi-dimensional MRI methods to study the etiology of various type of TBI, in collaboration with the Neuropathology Core of the CNRM.

We are also collaborating with Sara Inati, who has a dedicated clinical research program to study and diagnose focal epilepsy, a devastating disorder that is difficult to detect using conventional radiological methods. We are developing a bevy of new MRI–based methods that we believe may reveal pathological microstructural features in the disorder, for example, in cortical dysplasias.

**Biopolymer physics: water–ion–biopolymer interactions**

Water-ion-biopolymer interactions play a myriad of roles in biology. Our primary objective in this project is to understand such interactions in the biological milieu. Remarkably, little is understood about their physical underpinnings, particularly in their physiological ionic strength regime, despite their crucial role.

To determine the effect of ions on the structure and dynamics of key biopolymers, we developed a multi-scale experimental framework by combining macroscopic techniques (osmotic swelling pressure measurements, mechanical measurements) with scattering methods (e.g., SANS and SAXS), which probe the structure and interactions over a broad range of length and time scales. Macroscopic swelling pressure measurements provide information on the overall thermodynamic response of the system, while SANS and SAXS allow us to investigate biopolymers at molecular and supramolecular length scales and to quantify the effect of changes in the environment (e.g., ion concentration, ion valance, pH, temperature) on the structure and interactions among biopolymers, water, and ions. Studies carried out on well-defined model systems that mimic essential features of tissue provide important insights that cannot be obtained from experimental studies made on biological systems. Mathematical models based on well-established polymer physics concepts and molecular dynamics approaches make it possible to design and analyze fundamental experiments, which quantify and
explain aspects of tissue behavior in order to gain insight into the underlying molecular mechanism that governs key aspects of tissue structure-function relationships.

Better understanding of the structure and interactions among tissue components is also necessary to design and develop models and phantoms that mimic tissue behavior. Biomimetic phantoms with well-controlled physical properties (osmotic, mechanical, relaxation, etc.) are critically important to validate our quantitative MRI applications, from bench to bedside in our imaging studies. We produced novel diffusion MRI phantoms, which we use to calibrate MRI scanners, specifically to assure the quality of the imaging data and to assess scanner performance on an on-going basis. Our U.S. Patent for a “Phantom for diffusion MRI imaging” is now enabling quantitative diffusion MRI studies to be performed at many different sites. Our colleagues at NIST in Boulder, Colorado, have incorporated our PVP polymer into their own diffusion MRI NIST standard. The technology is also being promulgated commercially e.g., by High Precision Devices, Inc. We also developed a variety of NMR and MRI phantoms that possess various salient features of cell or tissue systems, providing ‘ground truth’ data with which to test the validity of our models and experimental designs.

**Measuring and mapping functional properties of extracellular matrix (ECM)**

Extracellular matrix (ECM) is present in every tissue and performs a myriad of roles in determining normal and abnormal tissue and organ function. We study interactions among the main ECM components, using cartilage as a model system. In cartilage ECM, collagen (type II) is organized into fiber bundles that form a network that entraps the major proteoglycan (PG), a bottlebrush-shaped aggrecan. The biomechanical behavior of cartilage and other ECMs reflects their molecular composition and microstructure, which change during development, disease, degeneration, and aging. To determine tissue structure/function relationships, we measure various physical/chemical properties of ECM tissues and tissue analogs at different length and time scales, using a variety of complementary static and dynamic experimental techniques, e.g., osmometry, SANS, SAXS, neutron spin-echo (NSE), SLS, DLS, and AFM. Understanding the physical and chemical mechanisms affecting cartilage swelling (hydration) is essential to predicting its load-bearing ability, which is mainly governed by osmotic and electrostatic forces. To quantify the effect of hydration on cartilage properties, we previously developed a novel tissue micro-osmometer to perform precise and rapid measurements on small tissue samples (less than 1 microgram) as a function of the equilibrium water activity (vapor pressure). We also make osmotic pressure measurements to determine how the individual components of cartilage ECM (e.g., aggrecan and collagen) contribute to the total load-bearing capacity of the tissue. We also demonstrated that aggrecan-hyaluronic aggregates self-assemble into microgels, contributing to improved dimensional stability of the tissue and to its lubricating ability. We also found that aggrecan is highly insensitive to changes in the ionic environment, particularly to calcium ions, which is critically important to maintaining the tissue’s mechanical integrity and to allowing aggrecan to serve as a calcium ion reservoir in cartilage and bone.

To model cartilage ECM, we recently invented and developed a new biomimetic material consisting of polyacrylic acid (PAA) microgel particles dispersed and embedded within a polyvinyl alcohol (PVA) gel matrix. In this novel composite hydrogel, PAA mimics the proteoglycan (i.e., hyaluronic-aggrecan complexes), while PVA mimics the fibrous collagen network entrapping them. Remarkably, the PVA/PAA biomimetic model system reproduces not only the shape of the cartilage swelling pressure curves, but also the numerical stiffness values reported for healthy and osteoarthritic human cartilage samples. Systematic studies made on these model composite hydrogels should yield invaluable insights into the effects of various macromolecular factors.
(matrix stiffness, swelling pressure, fixed-charge density, etc.) on the tissue’s macroscopic mechanical/swelling properties, and ultimately the origin of its load-bearing and lubricating abilities.

We are now attempting to translate this critical tissue-science understanding of the structure-function relationships of ECM components to develop and design novel non-invasive MRI methods, with the aim of inferring ECM composition, patency, and functional properties in vivo. Our goal is to use MRI for early diagnosis of diseases of cartilage and other tissue and organs, as well as to provide a means for following normal and abnormal ECM development, which entails making ‘invisible’ components of ECM, (e.g., collagen and PGs) ‘visible,’ and then using our understanding of biopolymer interactions to predict functional properties of the composite tissue, such as its load-bearing ability. One major obstacle is that protons, i.e., hydrogen atoms, bound to immobile species (e.g., collagen) are largely invisible using conventional MRI. However, magnetization exchange (MEX) MRI (as well as other methods) make it possible to detect the bound protons indirectly by transferring their magnetization to the free water protons surrounding them. It also enables us to estimate collagen content in tissue quantitatively. In pilot studies with Uzi Eliav and Ed Mertz, we applied the new MEX MRI method to determine the concentration and distribution of the main macromolecular constituents in bovine femoral-head cartilage samples. The results were qualitatively consistent with those obtained by histological techniques, such as high-definition infrared (HDIRI) spectroscopic imaging. The work was originally supported by a DIR Director’s Award to our collaborators Sergey Leikin and Edward Mertz, which they are continuing to pursue collaboratively. Our novel approach has the potential to map tissue structure and functional properties in vivo and noninvasively. In tandem, we are now developing continuum models of cartilage and cartilage ECM analogs in order to explain and interpret our experimental findings, develop and test novel hypotheses, and predict the behavior of our model system under different experimental conditions.

We also recently began using several novel one-sided NMR methodologies to study water relaxation, diffusion, and exchange behaviors in ECM as a means to infer and characterize its critical functional properties. Our specialized NMR scanner can probe layered media, such as cartilage, using ultra-thin slices, almost as thin as a confocal microscope provides. However, optical imaging is not a viable option, given that these tissues are turbid.

Patents

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Publications

Collaborators
- Ruijiang Bai, PhD, Zhejiang University, Hangzhou, China
- John Butman, MD, PhD, Radiology and Imaging Sciences, Clinical Center, NIH, Bethesda, MD
- Emilos Dimitriadis, PhD, Division of Bioengineering and Physical Science, NIBIB, Bethesda, MD
- Jack Douglas, PhD, NIST, Gaithersburg, MD
- Uzi Eliav, PhD, Tel Aviv University, Tel Aviv, Israel
- Dario Gasbarra, PhD, University of Helsinki, Helsinki, Finland
- Erik Geissler, PhD, CNRS, Université Joseph Fourier de Grenoble, Grenoble, France
- Mark Hallett, MD, PhD, Human Motor Control Section, NINDS, Bethesda, MD
- Iren Horkayne-Szakaly, MD, Joint Pathology Center, Armed Forces Institute of Pathology, Washington, DC
- Sara Inati, MD, Electroencephalography (EEG) Section, NINDS, Bethesda, MD
- Sergey Leikin, PhD, Section on Physical Biochemistry, NICHD, Bethesda, MD
- Edward L. Mertz, PhD, Section on Physical Biochemistry, NICHD, Bethesda, MD
- Gil Navon, PhD, Tel Aviv University, Tel Aviv, Israel
- Dzung Pham, PhD, CNRM Imaging Core, Henry M. Jackson Foundation, Bethesda, MD
- Carlo Pierpaoli, MD, PhD, Section on Quantitative Medical Imaging, NIBIB, Bethesda, MD
- Dietmar Plenz, PhD, Section on Critical Brain Dynamics, NIMH, Bethesda, MD
- Tom Pohida, MS, Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD
- Randall Pursley, Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD
- Evren Özarslan, PhD, Linköping University, Linköping, Sweden
- Joelle Sarlls, PhD, In Vivo NMR Center, NINDS, Bethesda, MD
- Brain Development Cooperative Group, Various

Contact
For more information, email peter.basser@nih.gov or visit http://sqits.nichd.nih.gov.
Biophysics of Large Membrane Channels

The Section studies mechanisms of channel-facilitated transport across cell and organelle membranes by combining experiments on channel reconstitution into planar lipid membranes with the physical theory, allowing for both qualitative and quantitative understanding of the transport processes. We focus mostly on large, metabolite-transporting channels such as VDAC (voltage-dependent anion channel from the outer membrane of mitochondria), OmpF (general bacterial porin from *Escherichia coli*), LamB (sugar-specific bacterial porin from *Escherichia coli*), OprF (porin from *Pseudomonas aeruginosa*), MspA (major outer-membrane porin from *Mycobacterium smegmatis*), alpha-Hemolysin (toxin from *Staphylococcus aureus*), and translocation pores of *Bacillus anthracis* (PA63), *Clostridium botulinum* (C2IIa), and *Clostridium perfringens* (Lb) binary toxins, Epsilon toxin (from *Clostridium perfringens*), Alamethicin (amphiphilic peptide toxin from *Trichoderma viride*), Syringomycin E (lipopeptide toxin from *Pseudomonas syringae*), and the bacterial peptide TisB involved in persister cell formation. We also use Gramicidin A (linear pentadecapeptide from *Bacillus brevis*) as a molecular sensor of membrane mechanical properties.

To investigate these mostly beta-barrel channels under precisely controlled conditions, we first isolate the channel-forming proteins from their host organisms, or use recombinant proteins, and then reconstitute them into planar lipid membranes. Healthy cell functioning and development require effective communication between cells and cell organelles, which is facilitated by membrane channels. Our main goal is to elucidate the physical principles of regulation of such channels under normal and pathological conditions. Among many wet-lab approaches, such as fluorescence correlation spectroscopy, bilayer overtone analysis, and confocal microscopy, single-channel electrophysiology is our hallmark technique that allows us to study transport processes at the single-molecule level. In our latest projects, we combine biophysical methods with those of cell biology. Empirical findings obtained in experiments are rationalized within the framework of a physical theory of channel-facilitated transport, which brings an understanding necessary to design new strategies to effectively correct the deviant interactions associated with disease.
FIGURE 1. Proposed model of the olesoxime neuroprotective effect

When alpha-synuclein (aSyn) is captured by the VDAC pore, it disrupts ATP/ADP fluxes through the VDAC. Under normal conditions, endogenous aSyn regulates the fluxes by reversibly and dynamically blocking the VDAC. Stress resulting from aSyn overexpression during Parkinson's disease induces aSyn translocation across the mitochondrial outer membrane (MOM) via the VDAC and targets electron transport chain complexes (ETC) in the inner membrane (MIM), causing their impairment, mitochondrial dysfunction, and eventually neuronal cell death. Olesoxime partitions into the MOM and hinders aSyn translocation through the VDAC by interacting with the pore-lipid interface. The model suggests a tentative mechanism of olesoxime protection of mitochondria integrity and promotion of neuronal cell survival.

Molecular mechanism of neuroprotective drug action through targeting alpha-synuclein interaction with mitochondrial VDAC

The second most common neurodegenerative disorder in the U.S. is Parkinson’s disease (PD), a neurological condition that causes severe movement problems. It is well known that the disease is associated with abnormal accumulation of a protein called alpha-synuclein, which is naturally produced in healthy neurons. Previous studies have shown that, when alpha-synuclein builds up in cells, it somehow moves through the mitochondrial outer membrane and targets respiratory complexes at the inner membrane, inducing mitochondrial dysfunction. However, the gateway that alpha-synuclein uses to enter mitochondria has long been a mystery. A hint to this puzzle appeared several years ago when our lab, using in vitro experiments with reconstituted mitochondrial VDAC, found that alpha-synuclein can translocate through this channel. This year, working in collaboration with Parkinson's disease researchers in the lab of Mark Cookson, we showed that VDAC is indeed a pathway for alpha-synuclein translocation into the mitochondria in cells. In this work, we studied effects of a cholesterol-like synthetic compound called olesoxime, which was previously shown to defend mitochondria from the alpha-synuclein–induced toxicity. Olesoxime generated considerable interest for its ability to protect neurons and mice in a range of neurodegenerative conditions, including PD. It was also established that olesoxime binds to VDAC in cells.

Following our interest in alpha-synuclein interaction with VDAC, we undertook to understand whether the molecular mechanism of olesoxime neuroprotection involves the interaction. Using neuronally differentiated human cells overexpressing wild-type alpha-synuclein as a cell model of PD, we found that olesoxime inhibits alpha-synuclein translocation into mitochondria. By applying a set of complementary electrophysiological and biophysical approaches, we provided mechanistic insights into the interplay between alpha-synuclein, VDAC, and olesoxime. Consistent with past studies, we found that overexpressed alpha-synuclein induces cell death and that olesoxime treatment dramatically reduced its rate by keeping mitochondria healthy. Working
at the single-molecule level, from the data obtained in channel-reconstitution experiments, we deduced that olesoxime interacts with the VDAC beta-barrel at the lipid-protein interface in such a way that it hinders alpha-synuclein translocation through the VDAC pore and affects VDAC voltage gating. The findings suggest that the alpha-synuclein interaction with VDAC is a new target for the development of drugs against alpha-synuclein toxicity. In particular, the use of molecules interacting with the alpha-synuclein–VDAC complex could be a promising and effective pharmacological treatment for a wide range of neurodegenerative conditions, such as spinal muscular atrophy, amyotrophic lateral sclerosis, Parkinson’s and Huntington’s diseases, and chemotherapy side effects, aimed to decrease mitochondrial deficiencies in affected neurons.

Effect of a post-translational modification mimic on protein translocation through a nanopore

Post-translational modifications (PTMs) of proteins are recognized as crucial components of cell-signaling pathways by modulating folding, altering stability, changing interactions with ligands, and therefore serving many regulatory functions. PTMs occur as covalent changes in the protein’s amino acid side chains or the length and composition of their termini. Inspired by the importance of PTMs in cell signaling, this year we studied the functional consequences of PTMs for alpha-synuclein interactions with mitochondrial VDAC. We mimicked PTMs of the 140-amino-acid cytosolic protein alpha-synuclein by attaching divalent Alexa Fluor 488 to the beginning and end of its C-terminal tail. Each of these modifications increases the total negative charge of the tail by two elementary charges and introduces extra bulkiness at the modification location, significantly changing the dynamics of the alpha-synuclein interaction with the VDAC pore. Using single-channel reconstitution into planar lipid membranes, we found that such PTM–like modifications change interactions drastically in both
the efficiency of VDAC inhibition by alpha-synuclein and its translocation through the VDAC pore. We analyzed time-resolved single-molecule events of alpha-synuclein capture by the VDAC pore within a framework of a one-dimensional diffusion model, using an interaction “quasi-potential” that incorporates mostly electrostatic and entropic components of alpha-synuclein interaction with the pore. The analysis proved to be an effective means of quantitatively describing the PTM–like modification effects on the kinetics of the capture, release, and translocation and yielded the positions of the modifications with an excellent precision of about three residues. Notably, the technique is general and can readily be applied to other biological channels and nanopores, suggesting that it could be extended to quantify populations of proteins that have undergone PTMs. Thus, in view of the recently established role of disordered charged termini of cytosolic proteins in the control of VDAC–facilitated transport, our findings establish a new mechanism of PTM–induced regulation of protein function.

Blocker escape kinetics from a membrane channel analyzed by mapping blocker diffusive dynamics onto a two-site model

Understanding the blockage of ion channels in biological membranes by natural and synthetic compounds is important from both theoretical and practical points of view. On the one hand, the phenomenon of blockage gives a rich example of the behavior of a solute molecule in strong confinement involving multiple interactions with the protein residues lining the channel walls and, in the case of a charged molecule, with the transmembrane electric field. On the other hand, its practical value is clear from the fact that about 13% of all present drugs of the world pharmacopeia act as ion-channel modifiers, with many of them working as channel blockers. This year, we developed an analytical theory of the blocker escape kinetics from the channel, assuming that a charged blocking molecule cannot pass through a constriction region (bottleneck). If the molecule spends a sufficiently long time in the channel, individual blockades in ionic current can be resolved in single-channel experiments. We focused on the effect of the external voltage bias on the blocker survival probability and characteristic times in the channel. The bias creates a potential well for the charged blocker, with the minimum located near the channel bottleneck. When the bias is strong, escape from the channel is a slow process, which allows for time-resolved observation of individual blocking events. We performed our
analysis in the framework of a two-site model of the blocker dynamics in the channel. Importantly, the rate constants, fully determining this model, were derived from a more realistic continuum diffusion model. This was done by mapping the latter onto its two-site counterpart which, while being much simpler, captures the main features of the blocker escape kinetics.

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- Colgate University award to William Rosencrans

**Publications**

**Collaborators**
- Vicente M. Aguilella, PhD, *Universidad Jaume I, Castellón, Spain*
- Alexandra Beilina, PhD, *Cell Biology & Gene Expression Section, NIA, Bethesda, MD*
- Alexander M. Berezhkovskii, PhD, *Division of Computational Bioscience, CIT, NIH, Bethesda, MD*
- Mark R. Cookson, PhD, *Laboratory of Neurogenetics, NIA, Bethesda, MD*
- Leonardo Dagdug, PhD, *Universidad Autónoma Metropolitana-Iztapalapa, Mexico City, Mexico*
- David Hoogerheide, PhD, *National Institute of Standards and Technology, Gaithersburg, MD*
- Jennifer C. Lee, PhD, *Biochemistry and Biophysics Center, NHLBI, Bethesda, MD*
- Ekaterina M. Nestorovich, PhD, *The Catholic University of America, Washington, DC*
- Sergei Y. Noskov, PhD, *University of Calgary, Calgary, Canada*
- Olga Protchenko, PhD, *Liver Diseases Branch, NIDDK, Bethesda, MD*
- Alexander Sodt, PhD, *Unit on Membrane Chemical Physics, NICHD, Bethesda, MD*
- Gerhard Wagner, PhD, *Harvard Medical School, Cambridge, MA*
- Michael Weinrich, MD, *Office of the Director, NICHD, Bethesda, MD*
- David L. Worcester, PhD, *National Institute of Standards and Technology, Gaithersburg, MD*
- Joshua Zimmerberg, MD, PhD, *Section on Integrative Biophysics, NICHD, Bethesda, MD*

**Contact**
For more information, email bezrukos@mail.nih.gov or visit http://smt.nichd.nih.gov.
Protein Sorting in the Endomembrane System

Our laboratory investigates the molecular mechanisms by which transmembrane proteins (referred to as “cargo”) are sorted to different compartments of the endomembrane system in eukaryotic cells. The system comprises an array of membrane-enclosed organelles including the endoplasmic reticulum (ER), the Golgi apparatus, the trans-Golgi network (TGN), endosomes, lysosomes, lysosome-related organelles (LROs, e.g., melanosomes), and various domains of the plasma membrane in polarized cells, such as epithelial cells and neurons (Figure 1). Transport of cargo between these compartments is mediated by vesicular/tubular carriers that bud from a donor compartment, translocate through the cytoplasm, and eventually fuse with an acceptor compartment. Work in our laboratory focuses on the molecular machineries that mediate such processes, including (1) sorting signals and adaptor proteins that select cargo proteins for packaging into the transport carriers, (2) microtubule (MT) motors and organelle adaptors that drive movement of the transport carriers and other organelles through the cytoplasm, and (3) tethering factors that promote fusion of the transport carriers to acceptor compartments. We study the machineries in the context of different intracellular transport pathways, including endocytosis, recycling to the plasma membrane, retrograde transport from endosomes to the TGN, biogenesis of lysosomes and LROs, autophagy, and polarized sorting in epithelial cells and neurons. We apply knowledge gained from this basic research to the elucidation of disease mechanisms, including congenital disorders of protein traffic, such as the pigmentation and bleeding disorder Hermansky-Pudlak syndrome (HPS), hereditary spastic paraplegias (HSPs), and other neurodevelopmental disorders.

The FHF complex mediates perinuclear distribution of AP-4 and its cargo ATG9A.

Over 20 years ago, we discovered the heterotetrameric adaptor protein complex 4 (AP-4) as a component of a protein coat associated with the TGN. Other groups then showed that mutations in AP-4 subunits cause a complicated form of autosomal-recessive hereditary spastic paraplegia termed AP-4-deficiency syndrome. Recent studies from our lab demonstrated that AP-4 mediates export of the transmembrane autophagy protein ATG9A from the TGN to pre-
autophagosomal structures, and that the export contributes to the maintenance of autophagic homeostasis. Failure to export ATG9A from the TGN in AP-4-deficiency patients may underlie the pathogenesis of the syndrome. This past year, we sought to identify additional proteins that cooperate with AP-4 in ATG9A trafficking. Using affinity purification and mass spectrometry, we identified the FHF complex (a protein complex thought to promote vesicle trafficking and/or fusion) as a novel AP-4 accessory factor. Knockdown of FHF subunits resulted in dispersal of AP-4 and ATG9A from the perinuclear region of the cell, consistent with the previously demonstrated role of the FHF complex in coupling organelles to the MT retrograde motor dynein-dynactin. The findings uncovered an additional mechanism for the distribution of ATG9A and provided further evidence for a role of protein coats in coupling transport vesicles to MT motors.

**Structure of human ATG9A, the only transmembrane protein of the core autophagy machinery**

The identification of ATG9A as an AP-4 cargo prompted us to examine the structure and function of the protein. ATG9A is the only transmembrane component of the core autophagy machinery and contributes to autophagosome biogenesis by mechanisms that are not well understood. In collaboration with the groups of Anirban Banerjee, Jiansen Jiang, and José Faraldo-Gómez, we succeeded in obtaining a 2.9 Å-resolution cryo-EM structure of human ATG9A. The structure revealed a novel fold with a homotrimeric domain-swapped architecture, multiple membrane spans, and a network of branched cavities, consistent with ATG9A being
a membrane transporter. In addition, structure-guided molecular simulations predicted that ATG9A causes membrane bending, explaining the localization of the protein to small vesicles and highly curved edges of growing autophagosomes.

Regulation of LC3B levels by ubiquitination and proteasomal degradation

In addition to working on ATG9A structure, we investigated the mechanisms of autophagy regulation. To this end, we conducted a genome-wide CRISPR-Cas9 knockout screen using cells expressing endogenous LC3B (a microtubule-associated protein that is central to the autophagy pathway) tagged with GFP-mCherry as a reporter, an approach that allowed us to identify the ubiquitin-activating enzyme UBA6 and the hybrid ubiquitin-conjugating enzyme/ubiquitin ligase BIRC6 as novel autophagy regulators. We found that the enzymes cooperate to mediate mono-ubiquitination and proteasomal degradation of LC3B, thus limiting the pool of LC3B available for autophagy. Depletion of UBA6 or BIRC6 raised the level of cytosolic LC3B, enhancing the degradation of autophagy adaptors and the clearance of intracellular proteins aggregates. The finding could be the basis for the development of pharmacological inhibitors of UBA6 or BIRC6 for the treatment of protein-aggregation disorders.

ARL8 relieves SKIP autoinhibition to enable coupling of lysosomes to kinesin-1.

The lab also focuses on the mechanisms that drive movement of organelles within the cytoplasm. Long-range movement of organelles relies on coupling to microtubule motors, a process that is often mediated by adaptor proteins. In many cases, the coupling involves organelle- or adaptor-induced activation of the microtubule motors by conformational reversal of an auto-inhibited state. This past year, we discovered that a similar regulatory mechanism operates for an adaptor protein named SKIP (also known as PLEKHM2). SKIP binds to the small GTPase ARL8 on the lysosomal membrane to couple lysosomes to the anterograde microtubule motor kinesin-1. Structure-function analyses of SKIP revealed that the C-terminal region, comprising three PH domains, interacts with the N-terminal region, comprising ARL8- and kinesin-1-binding sites. The interaction inhibits coupling of lysosomes to kinesin-1 and, consequently, lysosome movement toward the cell periphery. We also found that ARL8 not only recruits SKIP to the lysosomal membrane, but also relieves SKIP auto-inhibition, promoting kinesin-1–driven, anterograde lysosome transport. The findings demonstrate that SKIP is not merely a passive connector of lysosome-bound ARL8 to kinesin-1 but is itself subject to both intra- and inter-molecular interactions that regulate its function.

Synaptic vesicle precursors and lysosomes are transported by different mechanisms in the axon of mammalian neurons.

BORC is a multi-subunit complex that induces the recruitment of ARL8 to membranes. Previous work showed that BORC promotes coupling of mammalian lysosomes and *Caenorhabditis elegans* synaptic vesicle precursors (SVPs) to kinesins for anterograde transport of these organelles along microtubule tracks, raising the possibility that axonal lysosomes and SVPs are the same or related organelles. Analysis of wild-type and BORC-knockout mice, however, showed that SVPs and lysosomes are transported separately, and that only lysosomes depend on BORC for axonal transport in mammalian neurons. The findings thus demonstrated that SVPs and lysosomes are distinct organelles that rely on different machineries for axonal transport in mammalian neurons.
The Parkinson’s disease protein LRRK2 interacts with the GARP complex to promote retrograde transport to the TGN.

In an extension of our work on the role of the GARP complex as a TGN–tethering factor, we examined the physical and functional interactions of GARP with the protein leucine-rich repeat kinase 2 (LRRK2), in collaboration with the lab of Mark Cookson. Mutations in LRRK2 cause Parkinson's disease (PD); however, the precise function of LRRK2 remains unclear. We found that LRRK2 interacts with the VPS52 subunit of GARP (Golgi-associated retrograde protein). LRRK2 further interacts with the Golgi SNAREs (proteins that mediate vesicle fusion) VAMP4 and Syntaxin-6 and acts as a scaffold that stabilizes GARP-SNAREs complex formation. LRRK2 thus influences retrograde trafficking pathways in a manner that depends on its GTP-binding and kinase activity, an action that is exaggerated by mutations associated with PD and which can be blocked by kinase inhibitors. Disruption of GARP sensitizes dopamine neurons to mutant LRRK2 toxicity in Caenorhabditis elegans, showing that the pathways are interlinked in vivo.

Publications


Collaborators

- Anirban Banerjee, PhD, Unit on Structural and Chemical Biology of Membrane Proteins, NICHD, Bethesda, MD
- Mark Cookson, PhD, Laboratory of Neurogenetics, NIA, Bethesda, MD
- José Faraldo-Gómez, PhD, Theoretical Molecular Biophysics Laboratory, NHLBI, Bethesda, MD
- Jiansen Jiang, PhD, Laboratory of Membrane Proteins and Structural Biology, NHLBI, Bethesda, MD
- Yihong Ye, PhD, Laboratory of Molecular Biology, NIDDK, Bethesda, MD

Contact

For more information, email bonifacinoj@helix.nih.gov or visit https://www.nichd.nih.gov/research/atNICHD/Investigators/bonifacino.
Activity-Dependent Regulation of Neurons by the Neuregulin-ErbB Signaling Pathway

Failure of cortical microcircuits to properly regulate excitatory-inhibitory (E-I) balance is a key feature in the etiology of several developmental psychiatric disorders and neurological diseases, such as schizophrenia, autism, ADHD, and epilepsy. E-I balance is important to synchronize the firing pattern of local neuron ensembles, and its dysregulation can degrade cognitive functions and, in extreme cases, result in epileptiform activity. Network activity, in particular oscillatory activity in the gamma-frequency range (30–80 Hz), is altered in psychiatric disorders and may account for their cognitive and behavioral symptoms. We are interested in how Neuregulin and its receptor ErbB4, which are both genetically linked to psychiatric disorders, function in an activity-dependent fashion (i.e., experience) in the developing brain to regulate synaptic plasticity, neuronal network activity, and behaviors that, in rodents, model features of psychiatric disorders. We identified a functional interaction between Neuregulin/ErbB4, glutamatergic receptors (NMDARs), and dopamine (DA) signaling in GABAergic interneurons that is critical for understanding how Neuregulin can regulate E-I balance and synchronous activity in neuronal networks, processes that are important for cognitive functions altered in psychiatric disorders.

Our earlier studies demonstrated that, in the hippocampus and neocortex, expression of ErbB4, the major neuronal Neuregulin receptor, is restricted to GABAergic interneurons, particularly parvalbumin-positive (Pv⁺), fast-spiking interneurons, which are critically important for modulating gamma oscillation induction and power. Using genetically engineered mouse models, we discovered that Neuregulin-ErbB4 signaling regulates synaptic plasticity, neuronal network activity, and behaviors associated with psychiatric disorders. More recently, our group investigated other aspects of Neuregulin expression throughout the brain, its processing in response to neuronal activity, and its function in distinct neuronal populations of the developing and maturing nervous system. Distinct Neuregulin isoforms originate from four different genes (Neuregulin 1-4) or, in the case of the Neuregulin-1 gene, the use of alternative promoters. Neuregulin mRNAs encode proteins that have either one or two transmembrane (TM) domains. We recently discovered that, contrary to the widely accepted notion that all Neuregulins are targeted to axons to signal in juxtracrine or paracrine fashion, single-
pass TM Neuregulins are targeted to the soma of neurons and dual-pass TM Neuregulins are trafficked into axons (Figure 1). Furthermore, we uncovered a novel bidirectional signaling pathway in which single-pass TM Neuregulins are processed and released from the neuronal soma in response to excitatory transmission selectively through NMDARs, and then signal in autocrine fashion by activating ErbB4 receptors on GABAergic interneurons and promote internalization of NMDARs.

Our research uses a combination of *in vivo* and *in vitro* techniques that include: optogenetics, fiber photometry combined with genetically encoded calcium (GCaMP) and dopamine (dLight, GRAB) sensors to measure neuronal activity and neurotransmitter release, behavioral analyses that evaluate cognitive performance of wild-type and genetically modified mice using automated touchscreen systems, electrophysiological recordings in acute brain slices, confocal fluorescence microscopy of fixed and live tissues, proteomics analyses, and fluorescence-based multi-channel *in situ* hybridization (RNAscope, BaseScope). The ultimate goal of such multi-disciplinary approaches is to generate holistic models to investigate the developmental impact of genes that modulate E-I balance and neuronal network activity and that consequently influence behaviors and cognitive functions affected in psychiatric and neurological disorders.

**Subcellular distribution and functions of single- vs. dual-transmembrane Neuregulins in central neurons**

We recently found that single-pass transmembrane NRGs (sp-NRGs), such as NRG1 type II and NRG2, traffic as unprocessed pro-forms to the neuronal cell surface, where they accumulate at ER–PM junctions on neuronal somata and proximal dendrites. We had found that shedding of the signaling-competent ectodomain of

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**FIGURE 1. Subcellular NRG2 distribution in a cultured hippocampal neuron**

NRG2 (*white*) accumulates as an unprocessed pro-form on the cell body and proximal dendrites, where it forms puncta at contact sites between the plasma membrane and the endoplasmic reticulum. Axons’ initial segments are identified by Ankyrin G (*green*), dendrites by MAP2 (*purple*).
sp-NRGs is triggered by glutamatergic (i.e., excitatory) activity acting on NMDA receptors, and mediated by membrane-bound ADAM–type metalloproteinases. NMDA receptor activity promotes dissociation of unprocessed proNRGs from ER–PM contact sites through dephosphorylation of several conserved Ser/Thr residues located in their intracellular region and by ectodomain shedding by ADAM10 (Figure 2). Together, the two processes promote rapid, regulated release of biologically active sp-NRGs within minutes of NMDA receptor activation to promote ErbB4 signaling [Reference 1].

In stark contrast to sp-NRGs, dual-pass transmembrane (dp) NRG1 type III and NRG3 are targeted to axons and accumulate at glutamatergic presynaptic terminals onto GABAergic interneurons, where they signal in juxtacrine mode via postsynaptic ErbB4 receptors. Published and ongoing studies, primarily on NRG3, demonstrate that their unprocessed proforms are cleaved near the second TM domain by BACE-1 (an aspartic acid protease important for the formation of myelin sheaths in peripheral nerve cells), which is required for NRG3 to be released from the Golgi compartment and transported into axons (Figure 3).

**Neuregulin-2 and ErbB4 knockout mice exhibit dopamine dysregulation and severe behavioral phenotypes with relevance to psychiatric disorders.**

We found that NRG2 expression is more extensive than originally reported and includes striatal and medial prefrontal cortical (mPFC) neurons, while its receptor ErbB4 is expressed on projections from mesencephalic dopamine (DA) neurons that innervate these structures. To investigate the function of NRG2-ErbB4 signaling, we generated knockout (KO) mice for both. We found that NRG2 and ErbB4 KOs have higher extracellular DA levels in the dorsal striatum but lower levels in the mPFC and hippocampus, a pattern similar to DA dysbalance in schizophrenia. NRG2 and ErbB4 KO mice perform abnormally in a battery of behavioral tasks...
relevant to psychiatric disorders (Figure 4). They exhibit hyperactivity in a novelty-induced open field, deficits in prepulse inhibition, hypersensitivity to amphetamine, antisocial behaviors, reduced anxiety-like behavior in the elevated plus maze, and deficits in the T-maze alternation reward test, a task that is dependent on hippocampal and mPFC function. Acute administration of clozapine, a potent atypical antipsychotic, rapidly increases extracellular DA levels in the mPFC and improves alternation T-maze performance in NRG2 KO mice.

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<th>NRG2-/-</th>
<th>ErbB4-/-</th>
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<tr>
<td>Open field (Hyperactivity)</td>
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<tr>
<td>Elevated Plus Maze (Anxiety)</td>
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<td>Prepulse Inhibition (Sensorimotor gating)</td>
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<td>T-Maze (Working Memory)</td>
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<td>Amphetamine Sensitivity</td>
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FIGURE 4. Overlapping behavioral and neurochemical phenotypes in NRG2 and ErbB4 KO mice

Lack of either NRG2 or ErbB4 in genetically engineered mice elicits similar phenotypic alterations with relevance to psychiatric disorders, demonstrating that NRG2 is an important and non-redundant ErbB4 receptor ligand in the postnatal brain.

SECTION ON MOLECULAR NEUROBIOLOGY
FIGURE 5. Working memory deficits and reduced dopamine levels in KO mice are temporarily restored by clozapine administration.

A. Expression of NRG2 in the prefrontal cortex (PFC) analyzed by triple \textit{in situ} hybridization. NRG2 transcripts (white) are expressed in both glutamatergic (green) and GABAergic (magenta) neurons.

B. Poor performance by NRG2 KO mice in a T-maze reward alternation task, as compared with WT littermates (left), can be restored by administration of the antipsychotic drug clozapine (right).

C. Reduced extracellular dopamine levels in the mPFC of NRG2 KO mice.

D. Extracellular dopamine levels in the mPFC of NRG2 KO mice rise after clozapine injection (arrowhead) at a time that coincides with improved performance on the T-maze.

(Figure 5). The work emphasizes the importance of the NRG2-ErbB4 signaling pathway for proper function of the nigrostriatal, mesocortical, and mesolimbic DA systems [References 2 and 3].

\textbf{Differential analysis of ErbB4 function in mice harboring targeted mutations in either GABAergic or DAergic neurons}

Dysfunctional NRG-ErbB4 signaling in the hippocampus, PFC, and striatum may contribute to alterations in DA function associated with several schizophrenia symptoms. We previously reported that acute injections of NRG1 increase extracellular DA levels and regulate long-term potentiation (LTP) and gamma oscillations, and that ErbB4 is expressed in GABAergic (Pv+) and mesocortical DAergic (TH+) neurons. We therefore used genetic, biochemical, and behavioral approaches to measure DA function in the hippocampus, PFC, and striatum in mice harboring targeted mutations of ErbB4 in either Pv+ or TH+ neurons. Interestingly, we found that, in contrast to GABAergic neurons, ErbB4 is expressed DA neuron axons, and that NRG regulates extracellular DA levels by modulating dopamine transporter (DAT) function. In contrast to mice lacking ErbB4 in Pv+ GABAergic interneurons, which show sensory-motor gating deficits and increases in motor activity, mice lacking ErbB4 in TH+ DAergic neurons exhibit deficits in cognitive-related tasks in the T-, Y- and Barnes- mazes (Figure 6). Therefore, direct effects of NRG/ErbB4 signaling in GABAergic vs. DAergic neurons differentially affect cortical circuits and DA homeostasis and behaviors relevant to schizophrenia [Reference 4].

\textbf{A novel ultrasensitive \textit{in situ} hybridization (ISH) approach to detect short sequences and splice variants with cellular resolution}

Detection of short isoform-specific sequences typically requires RNA isolation for RT-PCR analysis, an approach that is not suitable to study their regional and cell-type–specific distribution. However, the ability to distinguish differential expression of RNA variants in tissue is highly desirable because alterations in mRNA
splicing and editing, as well as coding single-nucleotide polymorphisms, have been associated with numerous cancers as well as with neurological and psychiatric disorders. We reported on a novel highly specific and sensitive single-probe colorimetric/fluorescent ISH approach called BaseScope, which targets short exon/exon RNA splice junctions using single-pair oligonucleotide probes (50 bp), and which differs in several ways from the more traditional approach of RNAscope [Reference 6]. We used BaseScope to investigate, with single-cell resolution, the expression of four ErbB4 transcripts that arise from alternative splicing of exons encoding two juxtamembrane (JMa/JMb) and two cytoplasmic (Cyt-1/Cyt-2) domains (Figure 7). First, we validated the specificity and sensitivity of the approach by comparing ErbB4 hybridization with an exon 2–specific probe on brain sections from wild-type (WT) and ErbB4 KO mice lacking the exon 2. Next, we demonstrated that expression of ErbB4 JM/Cyt isoforms differs between neurons and oligodendrocytes. Based on these results, we propose that BaseScope could serve as an invaluable diagnostic tool to detect alternatively spliced isoforms, and potentially single-base polymorphisms, associated with disease [Reference 5].

Transduction of mesencephalic DAergic neurons via AAV–Cre microinjection can lead to off-target toxicity.

Stereotaxic microinjection of adeno-associated virus (AAV) expressing Cre recombinase (AAV–Cre) into specific brain regions has become a popular approach to ablate genes by loxP/Cre recombination in a regionally and temporally specific fashion. As part of our investigations into the functional role of the ErbB4 Cyt-1 isoform in DAergic neurons, we microinjected AAV–Cre into the ventral tegmental area (VTA) of mice harboring a conditional (floxed) ErbB4 Cyt-1 allele, using AAV–Cre titers commonly reported in the literature (about 10^{13} genome copies [GC]/mL). To our surprise, we found that this concentration is toxic to mesencephalic DA neurons. We furthermore found that the toxicity is independent of ErbB4 deletion, as AAV–Cre microinjection in both ErbB4 Cyt-1^{fl/fl} and WT C57Bl/6J mice resulted in similar losses of DA transporter (DAT) and tyrosine hydroxylase (TH) immunoreactivity, proteins critical for DA function, and elicited behavioral changes known to be associated with loss of DA neurotransmission. Furthermore, such phenotypes were observed with different AAV serotypes and proteins expressed (Cre/GFP mixture or Cre-GFP fusion protein). Interestingly, we observed that the AAV1 serotype affects DAT and TH expression, whereas AAV9 is toxic to DAergic neurons when used...
FIGURE 7. BaseScope analysis demonstrates that oligodendrocytes and neurons express distinct ErbB4 juxtamembrane (JM) transcripts.

The sensitivity and specificity of single-pair probe in situ hybridization was demonstrated by the presence of signals in sparse GABAergic hippocampal neurons of WT mice hybridized with a probe corresponding to exon 2 of the ErbB4 gene (A–C), and by the absence of signal in sections prepared from ErbB4 KO mice that lack exon 2 (D–F). Oligodendrocyte precursor cells (OPCs) and mature oligodendrocytes express ErbB4 JMa isoforms, which are susceptible to shedding and back-signaling (G,H), whereas GABAergic neurons express the cleavage-resistant JMb ErbB4 receptor (I,J).

at about $10^{13}$ GC/mL. Importantly, we found that diluting the viral titer 1000-fold to $10^{10}$ GC/mL effectively prevents the undesired effects while still efficiently recombining floxed targets. The work highlights the critical need for thorough and appropriate controls, such as including WT mice in experimental design, to account for potential off-target effects when working with AAV-Cre recombinase.

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- Center for Compulsive Behaviors Fellowship Award

**Publications**


**Collaborators**

- Veronica Alvarez, PhD, *Section on Neuronal Structure, NIAAA, Rockville, MD*
- Susan G. Amara, PhD, *Laboratory of Molecular and Cellular Neurobiology, NIMH, Bethesda, MD*
- Juan Bonifacino, PhD, *Section on Intracellular Trafficking, NICHD, Bethesda, MD*
- Jung Hwa (Susan) Cheng, PhD, *EM Facility, NINDS, Bethesda, MD*
- Jennie Garcia-Olivares, PhD, *Laboratory of Molecular and Cellular Neurobiology, NIMH, Bethesda, MD*
- Mario A. Penzo, PhD, *Unit on the Neurobiology of Affective Memory, NIMH, Bethesda, MD*
- Jung-Hoon Shin, PhD, *Section on Neuronal Structure, NIAAA, Rockville, MD*
- Hugo Tejeda, PhD, *Unit on Neuromodulation and Synaptic Integration, NIMH, Bethesda, MD*
- Judith R. Walters, PhD, *Experimental Therapeutics Branch, NINDS, Bethesda, MD*

**Contact**

For more information, email *buonanno@mail.nih.gov* or visit *http://smn.nichd.nih.gov*. 
Neuronal Circuits Controlling Behavior: Genetic Analysis in Zebrafish

The Section on Behavioral Neurogenetics studies how, under diverse environmental contexts, the nervous system selects appropriate behavioral responses to sensory information in a way that best satisfies internal motivational objectives. We use the larval zebrafish as a model because its brain exhibits the basic architecture of the vertebrate brain but is much less complex than the mammalian brain. Despite the relative simplicity of their nervous system, zebrafish have a sophisticated repertoire of sensory-guided and internally driven behaviors. Furthermore, the optical clarity of the embryo facilitates visualization of individual neurons and their manipulation with genetic techniques. Behavior in larvae is innate and therefore exhibits minimal variability between fish. Subtle alterations in behavior can therefore be robustly measured, making it possible to quickly assess the contribution of identified neurons to a variety of motor behaviors.

We focus on two aspects of behavioral regulation: the neuronal mechanisms by which sensory context regulates behavioral decisions and the pathways that sustain changes in behavioral state. Neuronal connections that allow the brain to integrate sensory and internal-state information are established through genetic interactions during development, and are frequently disrupted by gene mutations associated with neuro-developmental disorders. We can therefore use discoveries about sensorimotor integration pathways to understand how human disease genes disrupt brain development. To support these objectives, we develop new genetic tools and behavioral assays to probe the nexus between neuronal function and behavior at single-cell resolution.

Neuronal pathways for auditory sensory processing

Startle responses are rapid reflexes that are triggered by sudden sensory stimuli and which help animals defend against, or escape from, potentially threatening stimuli. In both fish and mammals, startle responses are initiated by giant reticulospinal neurons in the medulla, which receive short-latency sensory input from diverse sensory modalities. Although highly stereotyped, startle responses are nevertheless modulated by sensory context and behavioral state and are therefore an excellent system in which to understand how such information is integrated for behavioral choice. In mammals, including
humans, the startle response to a strong auditory stimulus can be inhibited by pre-exposure to a weak acoustic ‘prepulse’. This form of startle modulation, termed prepulse inhibition, is diminished in several neurological conditions. Our work in resolving the core neuronal pathway that mediates prepulse inhibition provides a basis to probe how gene mutations linked to neuro-developmental disorders disrupt sensory processing.

Analysis of startle control mechanisms is facilitated by using the zebrafish model. Sudden vibrational stimuli trigger rapid-escape swims in zebrafish, which are mediated by giant reticulospinal neurons, in a manner similar to the central neurons controlling startle responses in mammals. We previously demonstrated that escape swims are suppressed by pre-exposure to a prepulse, which allowed us to apply the powerful suite of genetic tools available in zebrafish to identify neurons that mediate prepulse inhibition. However, as in humans, zebrafish have more than one pathway for escape behavior. At larval stages, auditory stimuli drive either an extremely rapid Mauthner-cell mediated response, or a slightly delayed non-Mauthner behavior. Delayed responses are of special interest as they are not susceptible to prepulse inhibition; the effect of a weak prepulse is thus to increase use of delayed escapes while rapid escapes are suppressed. However, almost nothing was known about the neuronal pathway that mediates for delayed escapes. We therefore performed a circuit-breaking screen, in which we systematically ablated groups of Gal4–labeled neurons in a library of enhancer-trap lines, to identify neurons that are required to initiate delayed escape. We discovered a population of cells adjacent to the cerebellum in the prepontine hindbrain that initiate delayed escapes. Auditory cues that are insufficiently threatening to drive an immediate escape behavior activate the prepontine delayed escape population, allowing larvae to perform a flexible and graded response. The identification of such neurons provides a new paradigm for dissecting the circuit that mediates very rapid behavioral selection in an ethologically critical pathway [Reference 2].

**Neural mechanisms for behavioral state control**

Over the course of the day, motivational goals change in response to both internal and external cues. At a given moment, an individual’s behavioral state strongly influences decisions on how to interact with the environment. A major goal in neuroscience is to identify the neural systems that maintain short-term behavioral states and to determine how they interact with central mechanisms for behavioral choice.
In zebrafish, loss of illumination triggers a short-term behavioral state in which larvae show heightened locomotor activity. We previously demonstrated that light-sensitive neurons in the hypothalamus trigger this a state of hyperactivity and that the response to loss of illumination is a part of a light-search behavior.

Unexpectedly, we noted that during the first two minutes in the dark, zebrafish repeatedly turn in a single direction resulting in a circular swimming pattern, and that individual fish had a tendency to circle in a left or rightward direction (Figure 2). Left/right motor identity was stable across several days, and correlated also with left/right startle preference, when larvae were tested in the absence of overriding visual cues. Motor asymmetry is common across the animal kingdom and is manifested in humans as hand-preference. Although human handedness is known to be disrupted in several neuro-developmental disorders and has been the subject of much research, little is known about how it is generated during development, or maintained within the brain. We therefore used the opportunity to leverage our observation that zebrafish have an intrinsic motor asymmetry to investigate developmental and neural mechanisms.

To achieve this, we performed a circuit-breaking screen before testing for changes in motor bias. Two lines that labeled neurons required for motor bias both included a small group of around 60 cells in the posterior tuberculum, a forebrain region ventral to the thalamus. Unilateral laser ablation of posterior tubercular neurons imposed a motor bias on treated larvae, such that larvae with an intact left posterior tuberculum tended to circle rightward, and vice versa. We also found that an output pathway running through the habenula was required for such neurons to drive motor asymmetry. The experiments define the specific neuronal basis for motor asymmetry in zebrafish.

While conducting these studies, a spontaneous mutation occurred in our wild-type stock that strongly suppressed motor asymmetry. Using a high-throughput sequencing approach, we mapped the underlying
genetic lesion to a small deletion of the first two exons in the gene epb41l5, then used an independent mutation to confirm that fish with a heterozygous mutation in this gene fail to develop normal motor asymmetry. Given that epb41l5 interacts with members of the notch signaling pathway, we also tested left/right bias in heterozygous mutants with mib mutations, and again discovered loss of motor asymmetry. The findings indicate that normal acquisition of left/right motor identity is disrupted by gene mutations that quantitatively perturb Notch signaling levels during embryonic development [Reference 3].

Tools for decoding neuronal circuits
To enable circuit-breaking screens, we generated several hundred new Gal4 and Cre lines that can provide intersectional targeting of small groups of neurons with a high degree of specificity [References 4 & 5]. A unique feature of brain imaging in zebrafish is the ability to visualize the total architecture of the brain while simultaneously recording the position and morphology of every constituent labeled neuron. Thus, to make these tools accessible to the broader research community, we performed whole-brain imaging for each line, then registered the image of each line to the same reference brain. In collaboration with Nicholas Polys, we then developed an online brain atlas (http://zbbrowser.com) that enables researchers to quickly visualize the larval brain and locate transgenic lines to aid experiments. Such powerful visualization tools facilitate integrated analysis of reconstructed neuronal morphology in the context of the three-dimensional anatomy of the brain (Figure 3).

In order to build a brain atlas, we optimized a protocol that permits very high precision brain registration. We showed that such high precision of alignment permits statistically robust whole-brain analysis of neuronal composition and morphology in zebrafish models of neurological disorders. The technique can be applied to almost any zebrafish neuro-developmental model, thus enabling robust and quantitative detection of subtle changes in brain structure or composition. We recently used the method to test brain structure and composition in zebrafish that carry mutations in genes that are homologous to human genes known to be disrupted in autism-spectrum disorder. By combining analysis of changes in brain structure and startle
behavior in these mutants, we can define subsets of autism genes that have similar effects and are therefore likely to act in the same developmental pathways.

**Additional Funding**

**Publications**

**Collaborators**
- Ann M. Craig, PhD, *University of British Columbia, Vancouver BC, Canada*
- Sinisa Pajevic, PhD, *Mathematical and Statistical Computing Lab, CIT, NIH, Bethesda, MD*
- Nicholas Polys, PhD, *Virginia Polytechnic Institute and State University, Blacksburg, VA*
- Howard Sirotkin, PhD, *Stony Brook University, Stony Brook, NY*
- Lonnie P. Wollmuth, PhD, *Stony Brook University, Stony Brook, NY*

**Contact**
For more information, email *haroldburgess@mail.nih.gov* or visit *http://sbn.nichd.nih.gov*. 
Global Regulation of Bacterial Gene Expression by ppGpp

Our research goal is to understand the emerging fundamental regulatory functions in bacteria of the (p)ppGpp (guanosine pentaphosphate or tetraphosphate) alarmone, which is produced by RSH enzymes) and a new candidate alarmone, (p)ppApp (adenosine pentaphosphate or tetraphosphate), which are analogs of GTP, GDP, ATP and ADP. The (p)ppGpp pair are present in nearly all bacteria and many plant chloroplasts but, so far, have not been found in eukaryotes. The distribution of (p)ppApp is broad, but the details of its kinetic constants are uncertain. All appear to bind to E. coli RNA polymerase (RNAP), with (p)ppApp binding at a site opposite to the catalytic center of (p)ppGpp. Regulatory effects of (p)ppApp in E. coli in vitro suggest that the alarmone functions as an antagonist to (p)ppGpp. Our overarching objective is to learn, for both analogs, their enzymatic sources, functions, and whether there is regulatory cross talk. Current research efforts of many laboratories led to a consensus that (p)ppGpp functions as a first responder to most sources of physiological stress in bacteria, ranging from nutritional and physical to metabolic stress. Cellular defenses triggered by ppGpp operate to ensure bacterial adaptation and survival, including survival against host efforts to kill pathogens. We usually focus on E. coli as a model organism and on comparisons with other strains. Our past work and that of many others defined structural and regulatory features for both ppGpp–binding sites on RNAP, which, in the presence of high levels of ppGpp, can lead to activation as well as inhibition of nearly one third of the genome. Our first goal is to examine the spectrum of effects on cellular physiology, ranging from very modest to massive changes in (p)ppGpp basal levels. Subtle and intricate regulation responses result in almost imperceptible perturbations in amino-acid, carbon, lipid, or energy metabolism, which alter the balance between ppGpp synthesis and hydrolysis. Such behavior contrasts with effects of massive increases, or spikes, of ppGpp, which others recently associated with metabolic destabilization of ribosomes, rRNA, and tRNA, thereby requiring re-evaluation of a sixty-year central dogma, which we published in a recent review and which documents the rapid changes in the field [Reference 5]. The evidence that pathogenicity is enhanced by (p)ppGpp is now pervasive from the behavior of ppGpp⁰ cells that completely lack ppGpp. The profligate misuse of antibiotics coupled with very few new drugs makes fundamental studies of (p)ppGpp acutely relevant, if we are not to enter an era in which all antibiotics are ineffective.
(p)ppGpp is necessary and sufficient for initiation of chromosomal DNA synthesis.

*E. coli* growth can be determined by the different efficiencies of use of nutrients present in excess or by limiting abundance of efficiently used substrates, allowing cells to fine-tune gene expression, which contrasts starkly with starvation, where adjustments to prevent death are very different from the fine-tuning during growth. Classically, the Copenhagen school established that the range of balanced growth rates when nutrient efficiency is varied is correlated with changes in the cellular content of protein, RNA, and DNA: the faster the growth, the higher the macromolecular content. When ppGpp is deleted, the correlation is abolished: macromolecular content remains at the high levels typical of fast growth even during slow growth. The same is true for hydrolysis-suppressor mutants that elevate ppGpp under all growth conditions. Apparently ppGpp is necessary and sufficient for this phenotype, regardless of stress. RNAP suppressor-mutant phenocopies of *spotT* behave similarly in ppGpp0 backgrounds, indicating that the phenotype is mimicked entirely by transcriptional changes. Such an approach can establish that ppGpp not only determines growth rates but is also both necessary and sufficient to account for RNA and protein content changes.

This year, we published a paper [Reference 1] indicating that ppGpp0 strains can be used with DNA to reach a similar conclusion, i.e., that ppGpp is necessary and sufficient for chromosomal DNA initiation. Bacteria can divide as quickly as every 30 minutes, but it takes an hour to duplicate their circular chromosome. The disparity is resolved by multiple bidirectional initiation forks from a single origin (ori) region, which occur before DNA replication is completed at a single terminator (ter) sequence. Measurements of ori/ter DNA ratios by PCR provide accurate estimates of even small differences in initiation frequencies. It is also known that ppGpp mildly inhibits primase-mediated DNA elongation. Our genomic sequencing results reinforce ori/ter ratio differences for ppGpp0 strains and show that elongation inhibition sites are random without affecting ori/ter. The ori/ter ratios of ppGpp-containing cells drop about three-fold when comparing fast and slow growth, while the ori/ter ratios of ppGpp0 cells remain constantly high, even when their rate of balanced growth is slow. As before, parallel measurements of ori/ter ratios with suppressor mutants of ppGpp hydrolase and of RNAP mutants again led to the conclusion that ppGpp is necessary and sufficient for initiation of DNA replication.

While this work was under way, a report appeared proposing that ppGpp inhibition of ribosomal RNA operon (rrn) transcription from seven operons, comprising more than half of genomic transcripts, results in transmission of topological changes to the ori region that inhibit initiation. We tested the hypothesis with a strain deleted for all seven chromosomal rrn operons. The deletion strain remains viable because two high-copy plasmids carry either a single rrnB operon or several essential tRNA genes with the rrn deletions. Thus, indirect topological changes of ori/ter resulting from ppGpp inhibition of chromosomal rrn transcription are not possible. Inhibition of ori/ter ratios at slow growth rates persist in this strain as well as in its ppGpp0 derivative, a prediction not sustained by the hypothesis. A direct inhibitory mechanism is suggested by PCR, which shows three-fold more gyrase transcripts in wild-type than in ppGpp0 cells.

**Is (p)ppApp a regulator or a toxin in bacteria?**

The Potrykus lab approached this question by asking whether synthetic (p)ppApp has regulatory activity towards (p)ppGpp–sensitive *E. coli* promoters. Weak regulatory activities were encouraging, but the finding of strong specific binding near the RNAP catalysis center across from one of the (p)ppGpp binding sites was exciting and amplified by a similar sequence in *B. subtilis* RNAP. This led them to use bioinformatics to look for ppApp synthetase and a Mesh-1 like enzyme that could hydrolyze it. In *Methylobacterium extorquens* (Mex)
they found a (p)ppGpp and (p)ppApp synthetase present on a purified Rel-like protein catalytic fragment as well as a (p)ppApp hydrolase on the Mesh-like protein. Clearly, bacterial enzymes can synthesize (p)ppApp and ppGpp in vitro, providing the first biochemical proof that ppApp is the catalytic product of this enzyme in bacteria, as well as proof that (p)ppApp exists along with (p)ppGpp in both E. coli and B. subtilis [Reference 2]. Induced ectopic expression of RSHMex in E. coli led to appreciable (p)ppApp formation, providing evidence of its regulatory activity. Surprisingly, E. coli controls without ectopic RSHMex protein also revealed traces of (p)ppApp, constituting the first observation of native (p)ppApp in E. coli. Searches are now under way in E. coli for synthetase and hydrolase combinations that allow incremental accumulation of (p)ppApp or (p)ppGpp alone as well as together.

GreA is a well-studied RNA polymerase ancillary protein with complex functions, all involving ppGpp, that relate to fixing problems arising during transcription elongation arrest. The functions involve restoring proper reading frame and proof-reading when the polymerase slips out of phase. Katarzyna Potrykus discovered that GreA regulates its own synthesis by a unique stuttering mechanism, in a region termed GraL, rather than at a unique stop-site, during elongation [Reference 3]. GraL may be among the new functions found in a random mutant GreA protein library [Reference 4].

Publications

Collaborators
• Bozena Bruhn-Olszewska, PhD, University of Gdansk, Gdansk, Poland
• Katarzyna Potrykus, PhD, University of Gdansk, Gdansk, Poland
• Michal Sobala, PhD, University of Gdansk, Gdansk Poland

Contact
For more information, email cashel@mail.nih.gov or visit http://smr.nichd.nih.gov.
Membrane Rearrangements in Cell Fusion and Syncytin 1–Mediated Gene Transfer

Diverse biological processes in which enveloped viruses infect cells and cells from all kingdoms of life secrete, internalize, traffic and sort integral proteins, sculpt their membranes, and bring together parent genomes in sexual reproduction share a common stage: fusion of two membranes into one. Biological membrane remodeling is tightly controlled by protein machinery but is also dependent on the lipid composition of the membranes. Whereas each kind of protein has its own individual personality, membrane lipid bilayers have rather general properties manifested by their resistance to disruption and bending and by their charge. Our long-term goal is to understand how proteins fuse membrane lipid bilayers. We expect a better understanding of important fusion reactions to bring about new ways of controlling them and lead to new strategies for quelling diseases involving cell invasion by enveloped viruses and defects in intracellular trafficking or intercellular fusion. Our general strategy is to combine in-depth analysis of the best-characterized fusion reactions with comparative analysis of diverse, less explored fusion reactions, which can reveal new kinds of fusion proteins and clarify the generality of emerging mechanistic insights. In our recent studies, we explored the mechanisms of horizontal gene transfer from human cells transduced with retroviral vector to non-transduced cells.

Syncytin 1–dependent horizontal transfer of marker genes from retrovirally transduced cells

Retroviral transduction is routinely used to generate cell lines expressing exogenous non-viral genes. Retroviral vectors and cells containing retroviral vectors are considered for clinical applications. It is critically important to minimize the risk of the production of a replication-competent retrovirus (RCR) that may deliver the introduced gene or other genes from the transduced cell to non-transduced cells. To satisfy the latter requirement, the gene transfer plasmid lacks the genes required for γ-retroviral packaging and transduction. During production of a retroviral vector, such genes are provided by other plasmids or are stably expressed in the packaging cell line. Nevertheless, RCRs represent an important safety concern in the development of retroviral gene therapy.

In our recent study, we reported that human cells, transduced to stably express GFP, transfer the GFP gene to non-transduced cells. While this transfer was observed for many different types of donor (retrovirally...
transduced) cells to several different types of acceptor (non-transduced), the efficiency of the transfer depended on both donor cells and acceptor cells. GFP gene transfer was also observed when co-culture of the cells was replaced by application of the conditioned medium from the transduced cells.

Apparently, all mammalian cells release EVs. Given that, for retrovirus-releasing cells, the physical properties and sizes of many extracellular vesicles (EVs) strongly overlap with those of retroviruses, conventional EV preparations contain both EVs and retroviral particles and will be referred to below as extracellular membrane vesicles (EMV). Non-transduced PC3 cells incubated with EMVs from the cells retrovirally transduced to express GFP developed expression of GFP, with the fraction of the GFP–expressing cells similar to the fractions observed after co-culturing the non-transduced cells with the conditioned medium from the GFP–transduced cells.

We found that EMVs involved in the gene transfer carry endogenous the retroviral envelope protein Syncytin 1 and that the transfer depends on the fusogenic activity of Syncytin 1. Cell types expressing more Syncytin 1 are more efficient as donor cells, and suppression of Syncytin 1 expression in donor cells inhibits the transfer. A Syncytin 1–derived synthetic peptide known to block fusogenic restructuring of Syncytin 1, but not the control scrambled peptide, suppresses the GFP transfer by EMVs collected from the donor cells. The dependence of the gene transfer on the fusogenic activity of Syncytin 1 was further confirmed by inhibition of the transfer by blocking ASCT2 receptor of Syncytin 1 with an ASCT2–binding reagent ASCT2.

Overexpression of Syncytin 1 in the cells transduced with an envelope-defective retroviral sequence has been reported to result in assembly of pseudo-typed viruses with the ability to fuse with cells and to spread viral gene sequences to new target cells. Our findings indicate that even endogenous levels of expression of Syncytin 1 in cells can be sufficient for this protein to be acquired by EMVs and to effect Syncytin 1–mediated fusion of these vesicles. The levels of the expression of both Syncytin 1 and ASCT2 vary widely between different human cells and under different conditions. For instance, expression of Syncytin 1 and ASCT2 is boosted by the cytokines interleukins 4 and 13 and by tumor necrosis factor alpha. Several types of cancer, including prostate adenocarcinoma and endometrial carcinoma, have elevated levels of Syncytin 1 expression, with further upregulation of Syncytin 1 associated with cancer progression. Syncytin 1 expression is also boosted by some viral infections and linked to neurological diseases. Such data suggest that some retrovirally transduced cells can generate EMVs with Syncytin 1–dependent infectivity only under specific conditions, conditions that boost Syncytin 1 expression in these cells or ASCT2 expression in the surrounding cells.

In the context of clinical applications, where generation of RCRs is expected to increase the risk of treatment-related malignancy, emergence of RCRs is a very well-recognized concern. Both vector-producing cells and the transduced cells developed for gene therapy studies are routinely tested for the presence of RCR. Our findings indicate that stable cell lines generated by retroviral vectors in in vitro studies, where the transduction protocols are optimized to achieve the highest efficiency of the transduction by utilizing single packaging vector encoding gag, pol, and env, must also be tested for RCRs. Repetitive integration events that can accompany many infections by RCR can lead to important changes in protein expression profile and cell properties. Undetected and unexpected RCR production in laboratory cell lines presents an additional safety concern, especially given the fact that, in human cell line HERV, env proteins can change the tropism of the produced viral particles.
Lipid mixing assay for myoblast fusion and other slow cell-cell fusion processes

Lipid mixing (redistribution of lipid probes between fusing membranes) has been widely used to study early stages of relatively fast viral and intracellular fusion processes that take seconds to minutes. Lipid mixing assays are especially important for the identification of hemifusion intermediates, operationally defined as lipid mixing without content mixing. Given the unsynchronized character and the slow rate of the differentiation processes that prime the cells for cell-cell fusion processes in myogenesis, osteoclastogenesis and placentogenesis, such fusions take days. Application of lipid-mixing assays to detect early fusion intermediates in these very slow fusion processes must consider the continuous turnover of plasma membrane components and potential fusion-unrelated exchange of the lipid probes between the membranes. We applied a lipid mixing assay in our work on myoblast fusion stage in development and regeneration of skeletal muscle cells. Our approach utilizes a conventional in vitro model of myogenic differentiation and fusion based on murine C2C12 cells. When we observe the appearance of first multinucleated cells, we lift the cells and label them with either the fluorescent lipid DiI as a membrane probe or CellTracker™ Green as a content probe. Redistribution of the probes between the cells is scored by fluorescence microscopy. Hemifused cells are identified as mononucleated cells labeled with both content and membrane probes. The interpretation must be supported by a system of negative controls with fusion-incompetent cells to account for and minimize contributions of fusion-unrelated exchange of the lipid probes. With minor modifications the approach has been used to investigate fusion of primary murine myoblasts and osteoclast precursors, as well as fusion mediated by a gamete fusogen HAP2, and likely can be adopted for other slow cell-cell fusion processes.

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Publications


Collaborators

- Anush Arakelyan, PhD, Section on Intercellular Interactions, NICHD, Bethesda, MD
- Michael M. Kozlov, PhD, Dhabil, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel
- Leonid Margolis, PhD, Section on Intercellular Interactions, NICHD, Bethesda, MD
- Douglas Millay, PhD, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH
- Benjamin Podbilewicz, PhD, Technion-Israel Institute of Technology, Haifa, Israel

Contact

For more information, email chernoml@mail.nih.gov or visit https://www.nichd.nih.gov/research/atNICHD/Investigators/chernomordik.
Building the Zebrafish Lateral Line System

Cells divide, move, adhere, and interact with their neighbors and their environment to determine the formation of multicellular organ systems with unique fates, morphologies, function, and behavior. Our goal is to understand how such interactions determine the self-organization of cell communities in the nervous system of the zebrafish embryo. The lateral line is a mechano-sensory system that helps sense the pattern of water flow over the fish and amphibian body; it consists of sensory organs called neuromasts, which are distributed in a stereotypic pattern over the body surface. Each neuromast has sensory hair cells at its center, surrounded by support cells that serve as progenitors for the production of more hair cells during growth and for the regeneration of neuromasts. The development of this superficial sensory system in zebrafish is spearheaded by the posterior Lateral Line primordia (pLLp), groups of about 150 cells formed on either side of a day-old embryo near the ear. Cells in the primordia migrate collectively under the skin to the tip of the tail, as they divide and reorganize to form nascent neuromasts, which are deposited sequentially from its trailing end. Their journey is easily observed in live transgenic embryos with fluorescent primordium cells. Furthermore, a range of genetic and cellular manipulations can be used to investigate gene function and morphogenesis in the system. Understanding the self-organization of this relatively simple and accessible system in zebrafish will help elucidate the broader principles that determine cell-fate specification, morphogenesis, and collective cell migration in the developing vertebrate nervous system.

NetLogo Agent-based models as tools for understanding the self-organization of cell fate, morphogenesis, and collective migration of the zebrafish pLLp

Understanding how interactions between cells and their environment determine the self-organization of organ systems in a developing embryo is a central goal of developmental biologists. We used a combination of cellular, molecular, and genetic manipulations, coupled with multi-scale live imaging of the zebrafish embryo to understand the early development of the posterior Lateral Line (pLL). The relative simplicity of this sensory system, coupled with the ease with which
it can be imaged and manipulated during early zebrafish development, has led to its emergence in recent years as a model for understanding the broader principles of how signaling interactions within and between cells and their environment coordinate cell-fate specification, cell migration, morphogenesis and the self-organization of pattern and form in developing organ systems.

The Lateral Line is a sensory system that has evolved to help fish and amphibians detect the pattern of water flow over their body surface. It consists of sensory organs called neuromasts. Neuromasts have a relatively simple organization and display a characteristic distribution over the body surface. The pLL in zebrafish is a part of this sensory system, with neuromasts distributed on the lateral surface of the trunk and tail along a horizontal stripe that initially runs the length of what corresponds to the horizontal myoseptum. Each neuromast consists of a cluster of cells that come together to form an epithelial rosette. At the center of the rosette are sensory hair cells that extend out of the overlying skin and respond to being bent in a specific direction by water flow. Cells that surround the central sensory hair-cell progenitor are specified as support cells. They provide physical support to central sensory hair cells and also serve as a progenitor population that contributes to specification of additional sensory hair-cell progenitors, which divide to produce additional pairs of sensory hair cells during maturation and regeneration of the neuromasts.

Formation of the pLL system is spearheaded by the pLL primordium, which forms just under the skin as it separates from the pLL placode adjacent to the ear, leaving behind cells of the placode that will form sensory neurons of the pLL ganglion. Cells in the primordium initially have a compact but relatively mesenchymal morphology. However, a subset of the most trailing cells in the primordium begin to acquire distinct apical basal polarity, and they become taller as they assume a more epithelial morphology. These cells come together to form nascent neuromasts, or proto-neuromasts, as they apically constrict and reorganize to form an epithelial rosette around a central cell that is specified as a sensory hair-cell progenitor. After the first proto-neuromast forms at the trailing end, additional proto-neuromasts form sequentially closer to the leading end, as the primordium begins its journey under the skin toward the tip of the tail along the horizontal myoseptum. The most trailing cells in the primordium eventually lose their capacity to migrate, stop moving, and are deposited. Cells that were incorporated into epithelial rosettes are deposited as neuromasts, while surrounding cells that had not been incorporated into the rosettes are deposited individually as inter-neuromast cells between periodically deposited neuromasts. Though proliferation adds cells to the migrating primordium, cell loss owing to deposition from the trailing end exceeds the addition of cells due to proliferation, and the primordium progressively shrinks. Eventually, after depositing 5–6 neuromasts, it ends its journey by forming 2–3 terminal neuromasts near the tip of the tail.

Interactions between primordium cells and their environment determines the self-organization of the zebrafish pLLp as it migrates under the skin from the ear to the tip of the tail, forming and depositing neuromasts to spearhead formation of the pLL sensory system. In a paper published in the past year [Reference 1], we reviewed previously developed models that show how we used the NetLogo agent-based programming environment to visualize and explore how (1) self-generated chemokine gradients determine collective migration, (2) how progressive shrinking of a leading domain dominated by Wnt signaling (regulates cell-fate decisions) can be used to predict patterns of neuromast deposition, and (3) how NetLogo was used as a database for storing and visualizing the results of in toto lineage analysis of all cells in the migrating primordium. We also describe a new model that explores how previously defined interactions between Wnt and Fgf (potent regulators of cell proliferation and differentiation) signaling systems have the potential
to determine the periodic formation of center-biased Fgf signaling centers in the wake of a shrinking Wnt system. Together, the models illustrate how the programming environment can be used in diverse ways to integrate what has been learnt from biological experiments about the nature of interactions between cells and their environment, and to explore how such interactions could determine emergent patterns of cell-fate specification, morphogenesis, and collective migration of the zebrafish pLLp.

A sheath of motile cells supports collective migration of the zebrafish pLLp under the skin.

The zebrafish pLLp migrates in a channel formed by the underlying horizontal myoseptum and somites, as well as the overlying skin. While cells in the leading part of the pLLp are flat and have a more mesenchymal morphology, cells in the trailing part progressively reorganize to form the epithelial rosettes called proto-neuromasts. The epithelial cells extend basal cryptic lamellipodia in the direction of migration in response to both chemokine and Fgf signals. We showed that, in addition to the cryptic lamellipodia, the core tall epithelial cells are surrounded by a population of superficial flat motile cells, which extend actin-rich migratory processes apposed to the overlying skin. These thin cells wrap around the deeper proto-neuromasts, forming a continuous sheath of cells around the apical and lateral surface of the pLLp. The processes extended by such cells are highly polarized in the direction of migration, and such directionality, like that of the basal lamellipodia, depends on Fgf signaling. Consistent with interactions of sheath cells with the overlying skin contributing to migration, removal of the skin stalls migration, which is, however, accompanied by some surprising changes: there is a profound change in the morphology of the superficial cells, with directional superficial lamellipodia being replaced by undirected blebs or ruffles. Furthermore, removal of the skin not only affects lamellipodia of superficial cells, it simultaneously alters the morphology and behavior of the basal cryptic lamellipodia, even though the deeper cells that extend them do not directly contact the skin. Directional actin-rich protrusions on both the apical and basal surface and migration are completely and simultaneously restored upon regrowth of the skin over the pLLp. Embedding skin-removed embryos in Matrigel suppresses bleb formation and partially restores formation both superficial and basal lamellipodia. However, only the polarization of the basal lamellipodia is recovered, and the primordium remains unable to migrate robustly. Taken together, such data suggest that the pLLp coordinates collective migration by extending lamellipodia both basally, against the underlying tissue, and superficially, against the skin, and that the presence of the overlying skin is essential for pLLp migration. The manner in which confinement by the skin occurs remains unclear and a question for future studies. However, the suppression of blebs and re-appearance of lamellipodia, following the re-embedding of the skin-removed embryos in Matrigel, which is rich in components of the extracellular matrix (ECM), suggests that re-introduction of ECM components, potential substrates for lamellipodia, are key to their recovery. However, as Matrigel does not lead to recovery of polarized migration, it is likely that the overlying skin contributes in additional ways, including the potential of the skin to confine diffusible signals that serve as directional migratory cues for the primordium and/or the possibility that physical confinement provides mechanical constraints that contribute to migration.

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Publications


Collaborators

- Hari Shroff, PhD, *Laboratory of High Resolution Optical Imaging, NIBIB, Bethesda, MD*

Contact

For more information, email *chitnisa@mail.nih.gov* or visit *http://chitnislab.nichd.nih.gov*.
Molecular Genetics of Heritable Human Disorders

We conduct research to delineate the pathophysiology of and develop novel therapies for the two major subtypes of type I glycogen storage disease (GSD-I): GSD-Ia, and GSD-Ib. GSD-Ia is caused by a deficiency in the liver/kidney/intestine-restricted glucose-6-phosphatase-α (G6Pase-α or G6PC), and GSD-Ib is caused by a deficiency in the ubiquitously expressed glucose-6-phosphate transporter (G6PT or SLC37A4). G6Pase-α is an endoplasmic reticulum (ER) transmembrane protein that regulates intracellular glucose production by catalyzing the hydrolysis of G6P to glucose and phosphate. The active sites of G6Pase-α face into the ER lumen and depend on G6PT, another ER transmembrane protein, to translocate G6P from the cytoplasm into the lumen. To function, G6Pase-α must couple with G6PT to form a functional G6Pase-α/G6PT complex, which maintains interprandial glucose homeostasis. GSD-Ia and GSD-Ib patients manifest the common metabolic phenotype of impaired glucose homeostasis and the severe long-term complication of hepatocellular adenoma/carcinoma (HCA/HCC). There is no cure for either GSD-Ia or GSD-Ib. We generated animal models of both disorders, which are being exploited to both delineate the disease more precisely and develop new treatment approaches, including gene therapy. We also generated several efficacious G6Pase-α- and G6PT-expressing recombinant adeno-associated virus (rAAV) vectors and provided a proof-of-principle gene therapy in murine GSD-Ia and GSD-Ib, which is safe, efficacious, and appropriate for entering clinical trials. In 2018, our rAAV-G6PC/rAAV-co-G6PC vector (US patent #9,644,216; European patent #EP3074510) technology was licensed to Ultragenyx Pharmaceutical Inc. (Novato, CA), who are currently undertaking a phase I/II clinical trial for GSD-Ia (NCT03517085). The initial dose-escalation trial is currently in progress, and no safety issues have been reported to date.

Prevention of hepatic tumor initiation in murine GSD-Ia by gene therapy and activation of tumor-promoting pathways in HCA/HCC

As stated above, the hallmarks of GSD-Ia are impaired glucose homeostasis and a long-term risk of HCA/HCC. Currently, there is no therapy to address HCA/HCC in GSD-Ia. We have shown that rAAV-mediated G6PC gene transfer to young G6pc−/− mice prevents HCA.
development. It remains unclear whether G6PC gene transfer during tumor development in GSD-Ia can prevent tumor initiation or abrogate pre-existing tumors. We showed that treating liver-specific G6pc<sup>-/-</sup> (L-G6pc<sup>-/-</sup>) mice at the tumor-developing stage with rAAV-G6PC restored hepatic G6Pase-α expression, normalized glucose homeostasis, corrected defective hepatic autophagy, and prevented de novo HCA/HCC development. However, gene therapy could not restore G6Pase-α expression in the HCA/HCC lesions and failed to abrogate the pre-existing tumors. We showed that the expression of 11 β-hydroxysteroid dehydrogenase type-1, which mediates local glucocorticoid activation, is down-regulated in HCA/HCC lesions, leading to impairment in the glucocorticoid signaling that is critical for activation of gluconeogenesis. This suggests that down-regulation of local glucocorticoid action in the HCA/HCC lesions may suppress G6Pase-α restoration by gene transfer. Collectively, our data show that rAAV-mediated gene therapy can prevent HCA/HCC initiation in L-G6pc<sup>+/–</sup> mice at the tumor developing stage, but cannot reduce any pre-existing tumor burden. [Reference 1].

We examined the signaling pathways promoting HCA/HCC development in GSD-Ia. We showed that G6Pase-α deficiency leads to impaired hepatic autophagy, which leads to sustained accumulation of p62, an autophagy-specific substrate that can activate tumor-promoting pathways, including Nrf2, mTORC1, β-catenin, and YAP. Consistently, HCA/HCC lesions in G6Pase-α-deficient livers display increased accumulation of p62 aggregates and phosphorylated p62 along with activation of Nrf2, mTORC1, β-catenin, and YAP signaling. Compared with HCA, HCC lesions displayed higher expression of oncogenes and PKM2, which are critical for tumorigenesis. Our data show that persistent hepatic autophagy impairment activates several tumor-promoting pathways, which contribute to HCA/HCC development in GSD-Ia.

**An evolutionary approach to optimizing G6Pase– activity for gene therapy of GSD–Ia**

GSD-Ia is characterized by impaired glucose homeostasis with a hallmark hypoglycemia following a short fast. Previously, we developed rAAV vectors expressing either the wild-type (rAAV-G6PC) or codon-optimized (co) (rAAV-co-G6PC) human G6Pase-α. The rAAV-co-G6PC vector, which contains a 20% change in the native G6PC coding sequence but has a higher potency, is currently being used in a phase I/II clinical trial for human GSD-Ia (NCT 03517085), as mentioned above. While routinely used in clinical therapies, codon-optimized vectors may not always be optimal. Codon-optimization can impact RNA secondary structure, change RNA/DNA protein binding sites, affect protein conformation and function, and alter post-transcriptional modifications, which may reduce potency or efficacy. We therefore sought to develop alternative approaches that could improve the expression yet minimize the impact of sequence changes resulting from broad codon-optimization.

The human, dog, mouse, and rat G6Pase-α share 87–91% sequence identity. Intriguingly, in vitro expression assays have routinely shown that the canine G6Pase-α isozyme is significantly more active than human G6Pase-α. We therefore expanded our analysis to compare G6PC genes across the evolutionary tree, seeking potential codon changes that could enhance enzymatic activity of human G6Pase-α. We identified a Ser-298 to Cys-298 substitution naturally found in canine, mouse, rat, and several primate G6Pase-α isozymes, which, when incorporated into human G6Pase-α sequence, markedly enhanced enzymatic activity. Using G6pc<sup>–/–</sup> mice, we showed that the efficacy of the rAAV-G6PC-S298C vector (US patent #10,415,044; European patent #EP3236984) was three times higher than that of the rAAV-G6PC vector.

We had shown previously that restoring 3% of normal hepatic G6Pase-α activity in G6pc<sup>–/–</sup> mice prevents HCA/HCC development and that mice harboring less than 3% of normal hepatic G6Pase-α activity are at risk of tumor development. We also showed that G6Pase-α deficiency leads to hepatic autophagy impairment,
which can contribute to hepatocarcinogenesis. We therefore undertook a long-term (66-week) preclinical characterization of the rAAV-G6PC-S298C vector in GSD-Ia gene therapy. We showed that the increased efficacy of rAAV-G6PC-S298C allowed $G6pc^{-/-}$ mice treated with a lower dose of this vector to survive long-term. Our study also showed that the rAAV-G6PC-S298C vector, which contains a 2% change in the native $G6PC$ coding sequence, provides equal or greater efficacy to the codon optimization approach, offering a valuable alternative vector for clinical translation in human GSD-Ia [References 2 & 3].

**The signaling pathways implicated in impairment of hepatic autophagy in GSD-Ia**

G6Pase-α deficiency in GSD-Ia leads to impaired hepatic autophagy, a recycling process important for cellular metabolism and homeostasis. Autophagy can be regulated by several energy-sensing pathways, including SIRT1, FoxO, AMPK, PPAR-α, and mTOR. In 10–day old global $G6pc^{-/-}$ mice, hepatic autophagy impairment was attributed to activation of mTOR and inhibition of AMPK signaling. In other studies, using adult L-$G6pc^{-/-}$ mice, hepatic autophagy impairment was attributed to down-regulation of SIRT1 signaling independent of mTOR. Given that these studies used a different mouse model, the signaling pathways implicated in autophagy deficiency in young $G6pc^{-/-}$ mice remained unclear. We therefore sought to reconcile the reported differences and elucidate the various energy-sensing signaling pathways regulating autophagy in $G6pc^{-/-}$ mice over the first four weeks of life. We showed that impaired SIRT1, FoxO3a, AMPK, and PPAR-α signaling are responsible for autophagy impairment but that mTOR is minimally involved. Hepatic SIRT1 overexpression corrects defective autophagy, restores FoxO3a expression, improves AMPK signaling but fails to normalize impaired PPAR-α expression or metabolic abnormalities associated with GSD-Ia. Importantly, restoration of hepatic G6Pase-α expression in $G6pc^{-/-}$ mice corrects defective autophagy, restores SIRT1/FoxO3a/AMPK/PPAR-α signaling and rectifies metabolic abnormalities. Taken together, the data show that hepatic autophagy impairment in
GSD-Ia is mediated by down-regulation of SIRT1/FoxO3a/AMPK/PPAR-α signaling, with SIRT1 playing a major role [Reference 4].

Correction of metabolic abnormalities in a mouse model of glycogen storage disease type Ia by CRISPR/Cas9–based gene editing

The rAAV-G6PC vector used in the current phase I/II clinical trial is episomally expressed, and the long-term durability of expression in humans is currently being established. We therefore sought to explore the use of the CRISPR/Cas9 technology to correct a pathogenic GSD-Ia variant in its native genetic locus. The most prevalent pathogenic mutation identified in Caucasian GSD-Ia patients is G6PC-p.R83C, representing 32% of diseased alleles. Using the CRISPR/Cas9–based gene editing technology, we generated a GSD-Ia mouse disease model, the G6pc-R83C mouse homozygous for the G6PC-p.R83C mutation, and showed that G6pc-R83C mice manifest impaired glucose homeostasis mimicking that of human GSD-Ia. We then used a CRISPR/Cas9–based gene-editing system to treat newborn G6pc-R83C mice and showed that the treated mice grew normally to age 16 weeks without hypoglycemia seizures. The treated G6pc-R83C mice, expressing 3% or more of normal hepatic G6Pase-α activity, maintained glucose homeostasis, displayed normalized blood metabolites, and could sustain 24 hours of fasting. Taken together, we have developed a second-generation therapy in which in vivo correction of a pathogenic G6PC-p.R83C variant in its native genetic locus could lead to potentially permanent, durable, long-term correction of the GSD-Ia disorder [Reference 5].

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Publications


Collaborators

• Alessandra Eva, PhD, Istituto Giannina Gaslini, Genova, Italy
• Youngmok Lee, PhD, University of Connecticut School of Medicine, Farmington, CT
• Brian C. Mansfield, PhD, *Foundation Fighting Blindness, Columbia, MD*
• Matthew F. Starost, PhD, *Diagnostic & Research Services Branch, Division of Veterinary Resources, NIH, Bethesda, MD*
• David A. Weinstein, MD, MSc, *University of Connecticut School of Medicine, Farmington, CT*

**Contact**
For more information, email *chouja@mail.nih.gov* or visit *https://irp.nih.gov/pi/janice-chou*.
Chromatin Remodeling and Gene Activation

Aberrant gene regulation is the basis of many disease states. Our main objective is to understand how genes are activated for transcription in the context of chromatin structure. Chromatin is not merely a packaging system for DNA in eukaryotic cells; it also participates in gene regulation. The structural subunit of chromatin is the nucleosome, which contains nearly two turns of DNA coiled around a central core histone octamer. Nucleosomes are generally quite regularly spaced along the DNA, like beads on a string. Gene regulation involves either attenuation of the inherently repressive properties of nucleosomes to facilitate gene expression, or enhancement of those properties to ensure complete repression, events that are choreographed by DNA sequence-specific transcription factors (activators and repressors) and chromatin-modifying complexes. The latter can be divided into two groups: histone- or DNA-modifying enzymes that implement the “epigenetic code”, and ATP-dependent remodeling machines that move or displace nucleosomes. We are exploiting and developing high-throughput technologies to obtain genome-wide maps of nucleosomes, chromatin-remodeling complexes and RNA polymerase II in budding yeast to determine how chromatin organization is affected when genes are activated. The current objectives of our yeast studies are: (1) to determine the roles of the various chromatin-remodeling complexes (RSC, SWI/SNF, ISW1, ISW2, CHD1, Ino80C) in chromatin organization and gene expression, why there are so many different remodelers, and whether they are functionally redundant; our studies so far indicate that each remodeling enzyme makes a different contribution to chromatin organization; and (2) to test the hypothesis that nucleosomes control DNA accessibility and play a vital role in gene regulation by blocking promoters. We are also extending our studies of chromatin remodeling from yeast to higher organisms.

Many human diseases have been linked to chromatin remodeling enzymes and epigenetic modifications. For example, mutations in the gene encoding the hSNF5 subunit of the SWI/SNF ATP-dependent chromatin-remodeling complex are strongly linked to pediatric rhabdoid tumors. The CHD class of ATP-dependent remodelers has also been linked to cancer and autism. Cancer therapies and drugs aimed at epigenetic targets are being tested. A full understanding of chromatin structure and the mechanisms by which it is manipulated is therefore of vital importance.
FIGURE 1. DNA packaging in the nucleus: to what extent does chromatin compaction limit access to the DNA?

DNA is packaged into the nucleus by histones. The basic structural subunit of chromatin is the nucleosome core, which contains about 147 bp of DNA wrapped nearly twice around a central octamer of core histones. Nucleosomes are regularly spaced along the DNA like beads on a string; the intervening DNA is called the linker DNA and is bound by linker histone (H1). The beads-on-a-string fiber spontaneously condenses into a heterogeneous fiber of about 30 nm width. Genomic regions rich in repetitive elements form constitutive heterochromatin in all cells, in which the chromatin fiber is even more condensed. Facultative heterochromatin is formed on genes that should be permanently silent in a specific differentiated cell type. Heterochromatin is densely packed and darkly staining in the electron micrograph shown here. Euchromatin is less condensed (light staining) and contains active genes. We are interested in determining to what extent chromatin limits DNA accessibility. Figure adapted from Chereji et al. Genome Res 2019;29:1985-1995.

Chromatin remodeling and DNA accessibility

Gene activation involves the recruitment of a set of factors to a promoter in response to appropriate signals, ultimately resulting in the formation of an initiation complex by RNA polymerase II and transcription, events that coincide with the removal of promoter nucleosomes to create a nucleosome-depleted region (NDR). This observation led to the generally accepted model that promoter nucleosomes physically block transcript initiation, acting as repressors by preventing access to specific transcription factor binding sites. The nucleosome is a highly stable structure containing tightly wound DNA, which is largely inaccessible to sequence-specific DNA–binding proteins. Activation occurs if sequence-specific ‘pioneer’ transcription factors are present (proteins that bind to nucleosomal sites with high affinity), and/or if ‘classical’ transcription factors, which are normally blocked by nucleosomes, recruit ATP–dependent chromatin remodelers to move or evict promoter nucleosomes, thus facilitating initiation complex formation.

The ATP–dependent chromatin remodelers variously move nucleosomes along DNA, remove the histones altogether, or form arrays of regularly spaced nucleosomes. Examples include the SWI/SNF and RSC complexes, which remodel nucleosomes on genes and at promoters, and the CHD and ISWI complexes, which are often involved in determining nucleosome spacing. The Ino80C complex is unusual because it has both properties. We wrote a review describing the ATP–dependent remodelers and their roles in chromatin organization [Reference 1].
FIGURE 2. Accessibility of active and inactive promoter DNA in mouse liver cells

Mouse genes were divided into active and inactive genes, defined by the hypersensitivity of their promoters to DNase I, which correlates with transcription levels. Active genes have nucleosome-depleted promoters, which are more sensitive to digestion by DNase I. The promoters of such genes are aligned on their transcription start sites (TSS). The grey background indicates average nucleosome positions from MNase-seq data for the same cells. The fraction of the DNA accessible to the AluI restriction enzyme was calculated as a function of AluI concentration. The fraction cut by AluI reaches a limit value of about 32% at higher AluI concentrations in promoter-proximal regions, except at the nucleosome-depleted promoters of active genes, where it reaches a limit value of about 58%. For active genes, the fraction cut by AluI is higher in the linkers than inside nucleosomes (the AluI and nucleosome peaks are out of phase). Inactive gene promoters are not nucleosome-depleted and show no nucleosome phasing. However, they are accessible in about 32% of the cells, even though they are inactive. Figure adapted from Chereji et al. Genome Res 2019;29:1985-1995.

Chromatin may block access to the DNA at several levels: the nucleosome itself, the higher order coiling of the nucleosomal fiber, and further condensation of nucleosomal fibers into heterochromatin (Figure 1). We have begun to test the hypothesis that DNA accessibility is of major importance in regulating gene expression. To do this, we needed a fully quantitative measure of DNA accessibility. Accordingly, we used the restriction enzyme AluI as a probe of chromatin structure and as a proxy for transcription factors, given that both bind to specific DNA sequences. We measured the digestion rate and the fraction of accessible DNA accurately at all genomic AluI sites in budding yeast nuclei and in mouse liver-cell nuclei. We observed that mouse liver DNA is significantly more accessible than yeast DNA, which can be explained simply by recognizing that the linker DNA between nucleosomes is much more accessible than nucleosomal DNA and that nucleosomes are spaced farther apart in mouse chromatin, resulting in longer linkers than in yeast chromatin, data indicating that nucleosome spacing is a major determinant of accessibility. More remarkable is the fact that DNA accessibility is binary, such that each site is accessible in some cells (i.e., in a linker and cut by AluI) and essentially completely inaccessible in the remaining cells (i.e., nucleosomal and resistant to AluI). In fact, we found no sites that are accessible in every cell or inaccessible in every cell, an observation that indicates that nucleosome positioning is generally imperfect, even at promoters, such that nucleosomes never reliably block a specific site. For example, AluI sites in inactive mouse promoters are accessible in some cells, even though the gene is inactive, implying that the nucleosome is insufficient to block transcription factor binding in all cells and suggesting that the simple promoter nucleosome block model is incorrect (Figure 2). Surprisingly, in mouse nuclei, the relatively decondensed euchromatin (which contains mostly active genes) and the highly condensed
heterochromatin (which contains mostly inactive genes) have very similar accessibilities to AluI, suggesting that transcription factors could also penetrate heterochromatin. Overall, our observations suggest that DNA accessibility is not likely to be the primary determinant of gene regulation.

**Differential nucleosome spacing in neurons and glia**

Most eukaryotic cells have a characteristic average nucleosome spacing of about 190 bp, corresponding to a linker of about 45 bp. However, cortical neurons have a shorter average spacing of 165 bp, similar to that of the yeasts. The significance of this atypical global chromatin organization is unclear. In a collaboration with Doug Fields [Reference 2], we compared the chromatin structures of purified mouse dorsal root ganglia (DRG) neurons, cortical oligodendrocyte precursor cells (OPCs), and cortical astrocytes. We found that DRG neurons have short average spacing (165 bp), whereas OPCs (182 bp) and astrocytes (183 bp) have longer spacing. Most genes in all three cell types have a promoter chromatin organization typical of active genes: a promoter NDR flanked by regularly spaced nucleosomes. In DRG neurons, nucleosome spacing downstream of promoters is longer than expected from the genomic average, whereas nucleosome spacing in OPCs and astrocytes is similar to the global average for these cells. Thus, the atypical nucleosome spacing of neuronal chromatin does not extend to promoter-proximal regions. Although we gained some insight into the role of nucleosome spacing in different cell types, its significance remains unclear.

**A method for assessing histone surface accessibility genome-wide**

Almost all genomic methods focus on the DNA in chromatin. We have begun to study how histone accessibility might be altered in the cell in response to transcription. For example, there is some evidence in the literature that nucleosomes can undergo a major conformational change, exposing internal surfaces of the histone octamer. In a collaboration with Jeffrey Hayes, we developed a method to assess exposure of histone protein surfaces in yeast chromatin [Reference 3]. A histone amino acid residue on the octamer surface (or buried within the octamer) is substituted with cysteine, and its exposure is measured by reaction with a thiol-specific reagent. Nuclei are treated with biotin-maleimide and then the chromatin is digested to nucleosomes by micrococcal nuclease. Biotinylated nucleosomes are purified using streptavidin beads. Nucleosomal DNA from input and affinity-purified samples are sequenced and compared. Currently, we are assessing the exposure of several different histone surfaces within active and inactive chromatin.

**Role of the Ino80C chromatin remodeler at highly expressed genes in yeast**

We continued our collaboration with Alan Hinnebusch, publishing several papers together concerning the roles of the SWI/SNF and RSC chromatin remodelers in promoter nucleosome eviction. We have now studied the role of the Ino80 remodeling complex (Ino80C) in transcriptional activation [Reference 4]. We showed that Ino80C is important for promoter nucleosome eviction and transcriptional activation. Compared with SWI/SNF, Ino80C generally functions over a wider region, spanning the promoter-flanking nucleosomes and NDR, at genes highly dependent on its function. Nucleosome eviction defects in cells lacking the Ino80 ATPase subunit are frequently accompanied by reduced promoter occupancies of TATA–binding protein (TBP), and diminished transcription. We concluded that Ino80C acts widely in the yeast genome, together with RSC and SWI/SNF, to evict promoter nucleosomes and enhance transcription.

**Mechanisms preventing mislocalization of centromeric histone H3**

Correct localization of the centromeric histone H3 variant (Cse4 in yeast; CENP-A in humans) to centromeres
is vital for faithful chromosome segregation. Over-expression and mislocalization of CENP-A has been observed in many cancers and correlates with increased invasiveness and poor prognosis. In a collaboration with Munira Basrai’s lab, we investigated the mechanism by which yeast cells prevent mislocalization of centromeric H3. The Basrai Lab identified yeast genes required to prevent Cse4 mislocalization using a genetic screen [Reference 5]. They include two F-box proteins, Met30 and Cdc4, which interact and cooperatively regulate Cse4 proteolysis, preventing its incorporation into non-centromeric nucleosomes and consequent chromosome instability. The Dbf4-dependent kinase complex also plays a role in Cse4 proteolysis, probably through the Psh1 E3 ubiquitin ligase [Reference 6].

**Publications**


**Collaborators**

- Munira A. Basrai, PhD, Genetics Branch, Center for Cancer Research, NCI, Bethesda, MD
- Harold Burgess, PhD, Section on Behavioral Neurogenetics, NICHD, Bethesda, MD
- Douglas Fields, PhD, Section on Nervous System Development and Plasticity, NICHD, Bethesda, MD
- Chhabi Govind, PhD, Oakland University, Rochester, MI
- Jeffrey J. Hayes, PhD, University of Rochester Medical Center, Rochester, NY
- Alan G. Hinnebusch, PhD, Section on Nutrient Control of Gene Expression, NICHD, Bethesda, MD
- Philip R. Lee, PhD, Section on Nervous System Development and Plasticity, NICHD, Bethesda, MD
- Vasily M. Studitsky, PhD, Fox Chase Cancer Center, Philadelphia, PA

**Contact**

For more information, email clarkda@mail.nih.gov or visit http://clarklab.nichd.nih.gov.
Damaged DNA is a leading cause of many human diseases and disorders. We study the formation and resolution of RNA/DNA hybrids, which occur during DNA and RNA synthesis. Such hybrid molecules may lead to increased DNA damage but may also play critical roles in normal cellular processes. We are interested in how RNA/DNA hybrids are resolved and in the role that ribonucleases H (RNases H) play in their elimination. Two classes of RNases H, Class I and Class II, are present in most organisms.

Human patients with mutations in RNASEH1 exhibit a typical mitochondrial myopathy phenotype (muscular). Our studies were the first to show that RNase H1 is essential for the maintenance of mitochondrial DNA. Mice deleted for the Rnaseh1 gene arrest embryonic development at day 8.5 as a result of failure to amplify mitochondrial DNA. Aicardi-Goutières syndrome (AGS), a severe neurological disorder with symptoms appearing at or soon after birth, can be caused by defective human RNase H2. We are examining mouse models of AGS to gain insight into the human disorder. To understand the mechanisms, functions, substrates, and basic molecular genetics of RNases H, we employ molecular-genetic and biochemical tools in yeast and mouse models.

Differences between Class I and Class II RNases H

Many of our investigations over the years focused on RNase H1. RNase H1 recognizes the 2’-OH of four consecutive ribonucleotides, while the DNA strand is distorted to fit into a pocket of the enzyme. Thus, the enzyme requires more than one ribonucleotide for cleavage of RNA in RNA/DNA hybrids. In both eukaryotes and prokaryotes, RNases H1 consist of a single polypeptide. In contrast, RNase H2 is a complex of three distinct polypeptides in eukaryotes but a single polypeptide in prokaryotes. The catalytic subunit of the hetero-trimeric RNase H2 of eukaryotes is similar in its primary amino-acid sequence to the prokaryotic enzyme. RNase H2 can recognize and cleave both RNA/DNA hybrids and a single ribonucleotide or the transition from the ribonucleotide in the case of RNA–primed DNA synthesis (e.g., rrrrr/DDDD in DNA—italics indicate transition from ribonucleotide to deoxyribonucleotide) [References 1 & 2].
FIGURE 1. Two RNase H2 mutants with differential ribonucleotide excision repair activity reveal a threshold of rNMPs for embryonic development.

RNase H2 has two distinct functions: initiation of the ribonucleotide excision repair (RER) pathway by cleaving ribonucleotides (rNMPs) incorporated during DNA replication; and processing the RNA portion of an R-loop formed during transcription. An RNase H2 mutant that lacked RER activity but supported R-loop removal revealed that rNMPs in DNA initiate the p53-dependent DNA damage response and cause early embryonic arrest in the mouse. However, an RNase H2 AGS–related mutant with residual RER activity develops to birth. Estimations of the number of rNMPs in DNA in these two mutants define a ribonucleotide threshold above which p53 induces apoptosis. Below the threshold, rNMPs in DNA trigger an innate immune response.

We are currently examining HEK293 cells with mutations in the \textit{RNASEH2A} gene, which yield RNase H2 proteins with a preference for incision at single rNMPs embedded in DNA and poor activity toward R-loops.

Several types of RNA/DNA hybrid structure are formed, and they are processed differently. Simple RNA/DNA hybrids consist of one strand of RNA paired with one strand of DNA. The HIV–AIDS reverse transcriptase (RT) forms such hybrids when copying its genomic RNA into DNA. The RT also has an RNase H domain that is structurally and functionally similar to the class I cellular RNase H and is necessary for several steps of viral DNA synthesis. R-loop hybrids (three-stranded nucleic acid structures) have two separated DNA strands, with one hybridized to RNA while the other is in a single-stranded form. Such structures sometimes form during transcription and can lead to chromosomal breakage. However, they are also part of the normal process of switching (recombination) from one form of immunoglobulin to another, resulting in distinct isoforms of antibodies. Another form of hybrid are single or multiple ribonucleotides incorporated into DNA during replication [Reference 1]. The first two types of hybrid are substrates for class I and II RNases H. The third is uniquely recognized by type 2 RNases H.

**Dual activities of RNase H2; Aicardi–Goutièrè syndrome**

Eukaryotic RNases H2 recognize and resolve RNA hybridized or covalently attached to DNA (two chemically distinct structures) using the same catalytic mechanism for hydrolysis. RNase H2 mutations that reduce catalytic activity, or fail to properly interact with \textit{in vivo} substrates, cause Aicardi-Goutièrè syndrome (AGS). Mutations in seven genes are known to cause AGS, with more than 50% of AGS patients having mutations in any of the three subunits of RNase H2. We previously expressed (in \textit{Escherichia coli}) and purified human RNases H2 with mutations corresponding to several of those seen in AGS patients; one such mutation, \textit{RNASEH2A–G37S} (G37S), has significant loss of RNase H2 activity. Using the 3D structure of the human enzyme that we had determined, we could locate all known mutations in RNase H2 that cause AGS. The wide distribution of the mutations
suggests that modest changes in stability and interaction with other unknown proteins, as well as loss of catalysis, can all cause AGS. A mutation near the catalytic center of G37S found in some AGS patients results in low RNase H2 activity for both embedded ribonucleotides in DNA and RNA/DNA hybrids [Reference 1]. We are developing mouse models of AGS to clarify which defects are associated with each RNase H2 activity.

Mice bearing the G37S mutation in homozygous form are perinatal lethal, i.e., either dead at birth or die within a few hours of birth [Reference 1]. Mutations in another gene, TREX1, also cause AGS, and it has been shown that homozygous knockout (KO) mice are viable but die after a few weeks owing to a cardiomyopathy that can be prevented by blocking either an innate or adaptive immune response. In contrast, the G37S–mutant perinatal lethality and the fact that RNase H2 KO mice die during early embryogenesis suggest a more severe defect than that seen in TREX1–KO mice. Damaged DNA that finds its way into the cytoplasm can be sensed by the cGAS protein producing the small molecule cGAMP, which interacts with the Sting protein, an important protein for the DNA-sensing in the innate immune pathway. Mice that are homozygous for G37S and deleted for the cGAS or Sting genes are mostly perinatal lethal but no longer exhibit increases in ISGs (interferon-stimulated genes). Interestingly, a small fraction of the double G37S–Sting KO are viable, indicating only limited involvement of ISGs in perinatal lethality. Further studies are under way, which we expect will lead us to the cause of lethality.

To distinguish among the defects that persistent RNA/DNA hybrids and single ribonucleotides joined to DNA cause in vivo, Hyongi Chon, a former postdoctoral fellow, rationally designed a modified RNase H2 to make an enzyme unable to cleave single ribonucleotides embedded in DNA but that retained RNA/DNA hydrolytic activity. The mutant enzyme, which we call RED (ribonucleotide excision deficient), resolves RNA/DNA hybrids, which are substrates of both RNase H1 and RNase H2. Unlike the mouse and human RNases H2, RNase H2 activity is not required in the yeast Saccharomyces cerevisiae. Employing the ease of genetic mutation studies in yeast, we demonstrated that yeast producing the RNase H2RED enzyme acted in vivo by leaving embedded ribonucleotides (rNMPs) in DNA but was potent in removing RNA in RNA/DNA hybrids.

Embryonic lethality of mice Rnaseh2b–KO strains has been attributed to accumulation of rNMPs in DNA, but lethality could be the result of loss of RNA/DNA hydrolysis or a combination of both rNMP and RNA/DNA hydrolysis defects [References 1 & 2]. To distinguish among the possible causes of embryonic lethality, we generated a mouse that produces the RNase H2RED enzyme. Mouse embryonic fibroblasts (MEFs) derived from Rnaseh2RED mice have the same high level of rNMPs as seen in Rnaseh2b–KO MEFs [Reference 2]. Interestingly, the Rnaseh2RED mice die around the same time as the Rnaseh2b–KO mice. Therefore, lethality of the Knockout and RED RNase H2 mouse strains may be caused by increased rNMPs in genomic DNA. Rnaseh2G37S/RED embryos also arrest at approximately the same stage as Rnaseh2aRED/RED embryos because of better association of RNase H2RED than RNase H2G37S with DNA substrate containing embedded rNMPs. The result is important because some RNase H2–AGS patients have similar compound heterozygous mutations in which there may be a dominant mutated enzyme.

Our studies on RNase H2-RED have permitted us and others to assign specific substrates to each of the two activities and determine which functions are related to various phenotypes seen when RNase H2 is absent [References 1–5]. One of our goals is to produce an RNase H2 with robust incision at single rNMPs in DNA but with poor RNA/DNA hybrid cleavage (hybrid-defective [HD]) to complement the RNase H2-RED enzyme. We have identified amino-acid changes that appear to produce such RNase H2-HD and are in the early stages of studies in both cell cultures and mice.
Detection of a threshold of ribonucleotide tolerance in DNA for embryonic development

Embryonic development in the absence of RNase H2 exhibits defects as early as E6.5 to 7.5, the period of gastrulation in which cell numbers double every 4–5 h. We found evidence that this is indeed the cause of embryonic lethality [Reference 2]. We used mice with a separation of function in the RNase H2 enzyme (RNase H2RED) that retained RNA/DNA hydrolysis but was unable to remove rNMPs in DNA. Embryonic development was arrested at E10, the same day as seen for embryos with complete loss of both of RNase H2’s functions. Compared with complete loss of both functions, RNase H2RED/RED mouse embryo fibroblast cells have only about 65% rNMPs in DNA. A mouse (RNase H2G37S) with partial loss of both RNase H2 activities develops to birth and retains about 30% of the number of rNMPs in DNA compared with cells with complete loss of RNase H2. A compound heterozygous mouse, in which both RNase H2RED and RNaseh2G37S are present, retains 40% of rNMPs in cells lacking RNase H2. Embryos with complete loss of RNase H2 or that express RNase H2RED or RNase H2RED/G37S all exhibit a p53–dependent DNA–damage response. In contrast, mice with RNase H2G37S develop to birth with little or no p53–dependent DNA damage. Mice expressing RNase H4G37S weigh about 1,000 mg at birth, whereas the early-arrested embryos with no RNase H2 or defective RNases H2-RED or -RED/G37S weight only one to a few mg, an enormous difference. We conclude that a threshold of tolerance of rNMPs in DNA for embryonic development past E10 is exceeded in all mouse strains tested except in RNase H4G37S.

Human patients with the RNASEH2A G37S mutation have AGS. Although the patients with RNASEH2A G37S mutations are homozygous, similar to our RNase H2RED/G37S mice, some AGS patients are compound heterozygous, with each allele having a different mutation in the same RNase H2 gene. In vitro studies of mutant forms of RNase H2 reflect the properties of the RNase H2 mutant but may be unreliable for assessing the contribution of each of the two forms of RNase H2 when both are present in vivo. The strong effect on the stage of lethality of RNase H2RED/G37S embryos indicates a competition between the rNMP–active RNase H2G37S and the inactive RNase H2RED for some step in the removal of rNMPs. The protein-proliferating cell nuclear antigen (PCNA) is a critical component for removing rNMPs in DNA. We suggest that the competition between RNase H2-RED and RNase H2-G37S occurs when RNase H2 interacts with PCNA to repair rNMPs in DNA, rather than binding to rNMPs in DNA.

High density of unrepaired genomic ribonucleotides leads to Topoisomerase 1–mediated severe growth defects in absence of ribonucleotide reductase in Saccharomyces cerevisiae.

DNA polymerases incorporate rNMPs during replication and repair, and the rate of incorporation is affected by the ratio of deoxyribonucleotides (dNTPs)/ribonucleotide (rNTPs) in cells. We reduced the cellular pools of dNTPs in yeast by depleting Rnr1, the major catalytic subunit of ribonucleotide reductase (RNR), which converts ribonucleotides to deoxyribonucleotides, and observed an accumulation of genomic rNMPs in absence of RNase H2. Deletion of the genes for RNase H1 and the catalytic subunit of RNase H2 under Rnr1 depletion induced cell lethality, presumably because cells cannot survive the combination of high levels of rNMPs in DNA and persistent RNA/DNA hybrids, leading to replicative stress, DNA damage, and cell death. We further increased the load of rNMPs in genomic DNA by using DNA polymerases with higher propensity to incorporate rNMPs during DNA synthesis and observed that cells lacking both Rnr1 and RNase H2 suffer from severe growth defects, defects that are reversed in absence of Topoisomerase 1 (Top1), an enzyme that transiently breaks and reseals phosphodiester bonds in DNA, because processing of rNMPs by Top1, when RER is defective, induces mutations and genome instability. We concluded that, in cells lacking RNase H2,
and containing a limiting supply of dNTPs, there is a threshold of tolerance for the accumulation of genomic ribonucleotides that is tightly associated with Top1-mediated DNA damage.

Our studies on RNase H2-RED permitted us and others to assign specific substrates to each of the two activities and to determine which functions are related to various phenotypes seen when RNase H2 is absent [References 1–5]. One of our goals is to produce an RNase H2 with robust incision at single rNMPs in DNA but with poor RNA/DNA hybrid cleavage (Hybrid defective, HD) to complement the RNase H2-RED enzyme. We identified amino-acid changes that appear to produce such RNase H2-HD, and we are in the early stages of studies in both cell cultures and mice.

**Abasic substrates**

Incorporated rNMPs embedded in DNA could be converted to abasic sites in which the flanking dNMPs would be connected by a ribose phosphate rather than an rNMP. In collaboration with the Storici and Tell groups, we examined the abilities of eukaryotic RNases H2 to cleave substrates containing a single ribose abasic (only sugar phosphate) site in duplex DNA. Prokaryotic RNases HII, but not eukaryotic RNases H2, can recognize and cleave at rNMPs in duplex DNA. Little was known about abasic sites in RNA until, in collaboration with Cheung’s group, we discovered that abasic sites are present in RNAs of yeast and human cells, and likely in all organisms. The abasic sites are located in or near R-loops. The Cheung group had previously shown that MPG and APE1 interact with R-loops. MPG is a glycosylase that removes the base of DNA, and as reported in our papers with Cheung, or RNA, which in turn can be cleaved by the apurinic/apyrimidinic endonuclease 1 (APE1). Abasic sites in DNA are repaired by Apel excision using the complementary DNA strand as template for repair. RNAs have no template to correct for the absence of a base. The association of abasic sites with R-loops is intriguing and suggests that RNAs complexed with DNA are protected from RNase H activities, similar to stable R-loops present at transcription start sites of highly transcribed genes. In theory, the DNA of R-loops could serve as templates for repair of the abasic site, in which case the abasic site would survive longer than abasic sites in single-stranded RNA, giving the appearance of association of abasic sites with R-loops. However, binding of MPG and APE1 suggests that R-loops might be good substrates for MPG to generate the abasic sites.

**Publications**

Collaborators

- Frederic Chedin, PhD, University of California Davis, Davis, CA
- Vivian G. Cheung, MD, University of Michigan Life Sciences Institute, Ann Arbor, MI
- Yosuke Mukoyama, PhD, Laboratory of Stem Cell & Neuro-Vascular Biology, NHLBI, Bethesda, MD
- Keiko Ozato, PhD, Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD
- Francesca Storici, PhD, School of Biological Sciences, Georgia Institute of Technology, Atlanta, GA
- Gianluca Tell, PhD, Università degli Studi di Udine, Udine, Italy
- Kiyoshi Yasukawa, PhD, Kyoto University, Kyoto, Japan

Contact

For more information, email crouch@helix.nih.gov or visit http://sfr.nichd.nih.gov.
We are interested in mechanisms of genome maintenance and organization. During interphase, chromosomes are surrounded by the nuclear envelope (NE), which separates the nuclear and cytoplasmic compartments of the cell. The sequestration of chromosomes within the nucleus has profound consequences for almost all aspects of gene expression and cell function. Communication between the nucleus and cytoplasm occurs through conduits called nuclear pore complexes (NPCs), which are embedded in the NE and consist of about 34 proteins called nucleoporins (Figure 1). Beyond nucleo-cytoplasmic trafficking, nucleoporins are important for chromosome organization, transcriptional control, RNA processing, cell signaling, and cell-cycle control. Both nucleoporins and soluble components of the nuclear trafficking machinery also perform transport-independent functions in mitotic chromosome segregation. The involvement of nucleoporins in such diverse events offers the intriguing possibility that they might coordinate these processes with nuclear trafficking and with each other. Moreover, nucleoporin dysfunction has important clinical implications: nucleoporin genes are frequently misregulated in cancers, and nucleoporin mutations cause congenital defects, pediatric nephrotic syndromes, and premature ovarian insufficiency. Nucleoporins are critical viral targets, and their disruption contributes to neurodegenerative conditions, including amyotrophic lateral sclerosis, frontotemporal dementia, and Huntington's disease.

Our research studies nucleoporins, NPC-associated proteins (e.g., the SUMO pathway, spindle checkpoint proteins), and other components of the nuclear transport machinery (e.g., the Ran pathway) throughout the cell cycle. Our goal is to define their biochemical roles and how their dysregulation causes human disease. We took a multifaceted approach toward this question, using both CRISPR–based strategies in mammalian cells and fly (Drosophila melanogaster) developmental genetics.

Selective degradation of nucleo-cytoplasmic transport proteins and their interphase function
Understanding the activities of individual nucleoporins has been complicated by their multifaceted nature, abundance, and unusual stability. To overcome these issues, we employed strategies for
selective and rapid degradation of individual proteins within human tissue-culture cells (Figure 2). Specifically, we used CRISPR/Cas9 to construct cell lines in which sequences encoding auxin-induced degron (AID) domains are inserted into both alleles of targeted genes within cells that also stably express the Transport Inhibitor Response 1 (TIR1) protein. TIR1 promotes rapid, selective degradation upon addition of the plant hormone auxin. We frequently also add a fluorescent tag to the targeted proteins, allowing their degradation to be monitored visually as well as biochemically. We have been successful in developing cell lines that allow conditional depletions of nucleoporins associated with different regions of the NPC (Figure 1).

Our recent findings regarding the roles of nucleoporins during interphase address three issues. First, we investigated the role of individual nucleoporins in NPC assembly and stability. Our results indicate that different regions of the NPC can persist independently after depletion of individual nucleoporins, suggesting that the NPC is a surprisingly modular structure. Second, we investigated the role of individual nucleoporins in different nuclear trafficking pathways, an assessment that includes evaluation of nuclear protein import, protein export, and RNA export. We are able to differentiate the roles of individual nucleoporins and can specifically show that the TPR protein has a unique and important role in nuclear mRNA export via the TRanscription-EXport-2 (TREX-2) complex (see also below). Third, we investigated how organization of the nuclear transport machinery impacts development. In particular, we found that an evolutionarily controlled mechanism for association of Ran’s GTPase–activating protein (RanGAP) is critical for the development of *Drosophila*. Defining the mechanism through which individual nucleoporins contribute to each of these processes will allow us to better design future experiments examining nucleoporin function in human development and disease.

**Analysis of nucleoporins demonstrates that the NPC is a highly modular structure.**

NPCs are built from many copies of roughly 34 distinct nucleoporins. Models of the NPC depict it as a composite of several sub-domains, which have been named the outer rings, inner ring, cytoplasmic fibrils, and
nuclear basket. The outer-ring domains of the NPC are formed from the Y-complex, which contains nine core nucleoporins (SEH1, SEC13, NUP37, NUP43, NUP85, NUP96, NUP107, NUP133 and NUP160), with a tenth subunit (ELYS) required for chromatin recruitment. Other nucleoporins (NUP205, NUP188, NUP155, NUP93, NUP35) form the inner-ring structures. The distinct roles of individual nucleoporins and their functional interactions remain poorly understood. Moreover, NPCs undergo a disassembly-reassembly cycle during mitotic division, and a lack of tools for acute manipulation of individual nucleoporins has therefore precluded the study of their roles in maintaining structures within pre-existing pores without complications from disruption of NPC assembly.

We added AID tags and fluorescent moieties by homozygously targeting gene loci encoding Y-complex and inner-ring nucleoporins. Auxin addition resulted in a rapid loss of the targeted proteins in each case, without degradation of other nucleoporins. We anticipated that loss of any Y-complex member should result in complete destabilization of the outer rings. While this was true after depletion of NUP96 or NUP107, the loss of other Y-complex members surprisingly left the outer-ring lattice in place. The findings suggest that the outer-ring structure is remarkably resistant to perturbations once it is fully assembled, and show that its members are not of equivalent importance in sustaining its stability. Importantly, near-complete loss of the outer ring in NUP96-depleted cells did not cause collapse of the rest of the NPC, as demonstrated through immunostaining, live microscopy, and mass spectrometry. The remarkable persistence of inner-ring nucleoporins particularly indicated resilience of the NPC structure. Interestingly, depletion of the inner-ring nucleoporin NUP188 caused an NPC disassembly that was opposite to the profile after NUP96 depletion: inner-ring components were extensively displaced, while the components of the cytosolic fibrils, outer ring, and basket were largely unaffected. Also, there was a global reduction of almost all nucleoporins upon loss of NUP93. High-resolution scanning electron microscopy of residual NPCs after depletion of NUP96 or NUP93 further confirmed the status of these structures. Together, our results indicate that the inner and outer rings of the NPC form distinct and independent structures, and that NUP93 serves as an NPC lynchpin essential for them both (Figure 3).
After depletion of the inner ring or outer rings, we tested whether the residual structures remained functional for the import and export of a model nuclear transport substrate. Remarkably, there were only minimal changes in both nuclear import and export rates upon loss of NUP96 or NUP188. However, NUP93 depletion caused a complete block in nuclear transport in both directions, confirming that global disruption functionally disabled NPCs. Together, the results indicate that persistent inner ring or outer-ring structures could still act as conduits for vectoral nuclear trafficking and that these modules can support independent and redundant trafficking routes. Removal of both sets of structures forecloses all nuclear trafficking. Notably, the persistence of functional pores lacking a subset of canonical nucleoporins suggests that terminally differentiated cells might retain substantial nuclear trafficking even with divergent NPC composition. Differentiated cells might thus customize function through altered NPC composition, potentially modulating specific trafficking pathways or aspects of NPC activity such as gene regulation and post-translational protein modifications.

Roles of nucleoporins in gene expression and RNA trafficking
A series of evolutionarily conserved complexes are co-transcriptionally recruited to nascent mRNAs, facilitating their processing as well as escorting them to and through the NPC, actions that are functionally linked; a failure to perform any of them during mRNA biogenesis directly impacts both upstream and downstream events. A key player in mRNA maturation is the TRanscription and EXport 2 (TREX-2) complex. Loss of the TREX-2 complex leads to defects in mRNA export, similar to the phenotype observed after loss of major mRNA export receptor NXF1. The GANP subunit of TREX-2 localizes within the nucleus and associates with a NPC structure called the nuclear basket, which protrudes from the nucleoplasmic face of the NPC. In vertebrates, the nuclear basket comprises three nucleoporins (BSK-NUPs) called NUP153, TPR, and NUP50. BSK-NUPs have been implicated...
FIGURE 4. Anchorage of dmRanGAP to dmRanBP2 is required for development.

A. Schematic representation of RanGAP association to NPCs. In yeast, RanGAP (*aqua*) is not bound to the NPC (*top*). In vertebrates, RanGAP binds to RanBP2 (*red*) in a complex that also contains Ubc9 (*blue*) and SUMO1 (*purple, middle*). In flies, dmRanGAP binds directly to dmRanBP2 (*bottom*).

B. Cumulative step histogram of head eversion event during pupal stage. Late 3rd instar larvae (5–day old) were collected on day 0. While head eversion occurred robustly in controls (both *w1118* and heterozygous *ranbp2A* flies), no head eversion event was observed in homozygous *ranbp2A* flies.

C. Behavior analysis of pupal ecdysis. Heterozygous or homozygous *ranbp2A* flies were collected at the white pupal stage (APF: after puparium formation). The homozygous *ranbp2A* fly failed to undergo head eversion (white arrowhead) and air bubble translocation after 24 hours, and the air bubble occupied most of the pupa (black arrowhead) after 96 hours.

in numerous processes beyond protein import and export, including chromatin remodeling, control of gene expression, and protein modification, as well as mRNA processing and export.

It has been difficult to analyze discrete NPC functions in the absence of vertebrate BSK-NUPs; knockout of these genes is deleterious for organisms, and their depletion by RNAi requires extended incubations, potentially allowing the emergence of secondary phenotypes from prolonged NPC disruption or defective post-mitotic NPC re-assembly. We used the auxin-mediated degradation system to untangle the functions of individual BSK-NUPs in both nuclear basket architecture and gene expression. We found that NUP153 and TPR bound to the NPC independently of each other and that loss of individual BSK-NUPs did not destabilize the NPC. We further found that TPR, but not NUP153 or NUP50, tethers the TREX-2 complex to the NPC. Loss of NUP50, NUP153, or TPR led to unique transcriptomic responses. Importantly, transcriptomic signatures after loss of TPR were more pronounced and were similar to changes upon the loss of either the GANP subunit of the TREX-2 complex or the RNA–export receptor NXF1. Moreover, similar to the case of NXF1 or GANP, loss of TPR led to retention of both upregulated and downregulated mRNA transcripts within the nucleus.

Taken together, such data support a unique role of TPR in transcription regulation and mRNA export through the TREX-2 complex. The findings both support our hypothesis that individual nucleoporins have distinct and non-redundant cellular functions and demonstrate the utility of the AID system to analyze their unique roles within cells.
Impact of Ran pathway organization beyond nuclear trafficking: development and mitosis

The Ran GTPase is a critical regulator of nuclear trafficking in eukaryotic cells. Ran's cytoplasmic GTPase-activating protein, RanGAP, and its chromatin-bound nucleotide exchange factor, RCC1, establish compartmental patterns for Ran's nucleotide-binding status: GTP-bound Ran (Ran-GTP) is abundant in the nucleus while GDP-bound Ran (RanGDP) is abundant in the cytosol. Such asymmetry drives nucleo-cytoplasmic trafficking through controlled cargo binding and release by karyopherins, a family of RanGTP-binding transport receptors.

In multicellular organisms, RanGAP is tethered to the cytoplasmic side of the NE, but the functional consequences of its localization had not been clarified. To investigate the importance of RanGAP association to the NE, we used human tissue-culture cells and Drosophila. In mammals, RanGAP tethering is mediated by its covalent conjugation to SUMO1, a small ubiquitin-like protein. We made human tissue-culture cells in which RanGAP was neither SUMOylated nor localized to the NE. Surprisingly, the cells showed neither obvious changes in viability nor substantial defects in nucleo-cytoplasmic transport of a model substrate. In Drosophila, we found that SUMOylation of RanGAP did not control its association with the NE. Rather, we identified a specific region within the nucleoporin dmRanBP2 that directly binds dmRanGAP, tethering it to the NPC (Figure 4). A dmRanBP2 mutant lacking this region showed no apparent growth defects during larval stages, but arrested at the early pupal stage. The developmental arrest was rescued by a direct fusion of dmRanGAP to the dmRanBP2 mutant, indicating that recruitment of dmRanGAP to dmRanBP2 per se was necessary for the pupal ecdysis sequence during development. Collectively, the results indicate that, while the localization of dmRanGAP to the NE is widely conserved in multicellular organisms, the targeting mechanisms are not. Furthermore, the localization appears to be critical for developmental processes rather than for viability at the cellular level. We continue to explore the developmental consequences of releasing the interaction of dmRanGAP to dmRanBP2.

We have a long-standing interest in the process of chromosome segregation. The Ran GTPase pathway and nucleoporins promote chromosome segregation through their important roles in spindle assembly and cell-cycle progression. After mitotic NE breakdown, RCC1 generates RanGTP near chromosomes, while Ran distal to chromosomes is GDP-bound, directing spindle assembly through spatially regulated release of spindle assembly factors (SAFs) from karyopherins. To segregate chromosomes accurately, RanGTP distribution must be tightly regulated, both spatially and temporally. Defects in chromosome segregation lead to aneuploidy, a condition in which cells possess an abnormal number of chromosomes. The error-prone nature of Ran-dependent spindle assembly is particularly important during meiosis, potentially contributing to human pregnancy losses and genetic disorders, including Down's syndrome. Moreover, aneuploidy arising from mitotic divisions is a hallmark of many solid tumors.

RanBP1 is a RanGTP-binding protein that forms a stable heterotrimeric complex with Ran and RCC1 in vitro (RRR complex), inhibiting RCC1's nucleotide exchange activity. We previously reported that RRR complex formation determines RCC1's partitioning between its chromatin-bound and soluble forms in embryonic systems, where RCC1 levels are very high, as well as specifically inhibiting the activity of soluble RCC1. Somatic cells have less soluble RCC1 during mitosis, raising questions about whether RRR complex formation is important after early development. To investigate the mitotic role of the RRR complex after early development, we examined whether RanBP1 or a related protein called RanBP3 might be important for controlling mitotic RanGTP gradients within somatic cells. We systematically varied RanBP1 and RanBP3 levels in HCT116 or
FIGURE 5. Regulation of mitotic Ran-GTP gradients by the RRR complex

Immuno-staining with HURP and Tubulin antibodies of cells in HCT116<sup>RCC1-µAID-3xFLAG</sup>, HCT116<sup>RanGAP1-3mAID</sup>, and HCT116<sup>RanBP1-µAID-HA</sup> cells treated with or without 1 mM Auxin for 5, 2, and 3 h, respectively. Cells also express TIR1. White dashed lines represent the length of HURP signal (top row).

Scale bars = 10 μm.

DLD1 cell lines through overexpression or fusion with AID tags. Consistent with earlier reports, RanBP1 was dispensable for interphase import or export of a model substrate, while RanBP3 appears to facilitate nuclear export via the Crm1 karyopherin. Within mitosis, altering RanBP1 levels substantially altered RCC1 dynamics on metaphase chromosomes, while altering RanBP3 levels did not. Moreover, we found dramatic re-localization of the SAF Hepatoma Up-Regulated Protein (HURP, a component of the spindle-assembly pathway) during metaphase in direct correspondence with changes in RCC1 dynamics (Figure 5), showing that RanGTP levels and SAF activity near chromosomes correlate with altered RCC1 behavior. Analogous to findings in embryonic systems, the data indicate an important mitotic role in human somatic cells for RanBP1 in controlling RCC1 dynamics and determining the accurate spatial distribution and magnitude of Ran-GTP gradients, thus ensuring correct execution of Ran–dependent mitotic events.

The role of the IRBIT protein in tissue homeostasis

We previously reported a conserved role for the IRBIT protein (IP3-receptor–binding protein released with inositol 1,4,5-trisphosphate) in inhibiting ribonucleotide reductase (RNR), an enzyme that produces deoxynucleotide triphosphates (dNTPs) for DNA synthesis. We further found that mammalian tissue-culture cells show altered cell-cycle progression and genome stability in the absence of IRBIT, and that the mechanism is conserved between humans and <i>Drosophila</i>. Therefore, in collaboration with Mihaela Serpe and Brian Oliver, we used flies as a model organism to understand the role of IRBIT in development and tissue homeostasis.

In situ hybridization showed IRBIT expression in regions destined to become the midgut during embryogenesis, and IRBIT is highly expressed in the adult midgut. The <i>Drosophila</i> midgut has a tubular structure and is surrounded by visceral muscles. The adult midgut possesses a monolayered epithelium that is composed of four distinct cell types (Figure 6B): intestinal stem cells (ISCs), undifferentiated progenitor cells called enteroblasts (EBs), specialized absorptive enterocytes (ECs), and secretory enteroendocrine cells (EEs). The
midgut is maintained through division of ISCs, giving rise to EBs, which in turn differentiate into EEs. Nutrients are absorbed from the lumen of the gut, which also contains a complex microbiota; the midgut acts both as a niche for commensal microbes and as the first line of defense against microbial pathogens. Like the intestine of vertebrates, the epithelium of the midgut has a remarkable regenerative capacity, which has been extensively exploited for the study of stem cell–driven tissue self-renewal, as well as tissue homeostasis during aging.

We examined IRBIT’s role in the midgut by generating an IRBIT null fly (IRBIT−/−) (Figure 6A). The midguts of one-day-old wild-type and IRBIT−/− flies were essentially indistinguishable at the tissue-architecture level. However, we observed a rapid and progressive increase in the fraction of Escargot-positive cells in the IRBIT−/− flies over time. In conjunction with additional experiments, the accumulation is indicative of accumulation of undifferentiated enteroblast progenitor cells.


A. Epithelia from control (left) and IRBIT−/− (right) flies at three (top) or seven (bottom) days after eclosion. The guts were stained with DNA dye Hoechst 33258 (blue), and antibodies against Prospero (to identify EE cells) and Escargot (to identify EB and ISC cells). We observed a rapid and progressive increase in the fraction of Escargot-positive cells in the IRBIT−/− flies over time. In conjunction with additional experiments, the accumulation is indicative of accumulation of undifferentiated enteroblast progenitor cells.

B. Schematic of epithelium within the Drosophila midgut. EC: enterocyte; ISC: intestinal stem cell; EB: enteroblast; EE: enteroendocrine cell.

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Collaborators

• Yoshiaki Azuma, PhD, University of Kansas, Lawrence, KS
• Beatriz Fontoura, PhD, University of Texas Southwestern Medical Center, Dallas, TX
• Amnon Harel, PhD, Azrieli Faculty of Medicine, Bar-Ilan University, Safed, Israel
• Natalay Kouprina, PhD, Developmental Therapeutics Branch, NCI, Bethesda, MD
• Vladimir Larionov, PhD, Developmental Therapeutics Branch, NCI, Bethesda, MD
• Brian C. Oliver, PhD, Laboratory of Cellular and Developmental Biology, NIDDK, Bethesda, MD
• Mihaela Serpe, PhD, Section on Cellular Communication, NICHD, Bethesda, MD

Contact

For more information, email dassom@mail.nih.gov or visit http://sccr.nichd.nih.gov.
Regulation of Mammalian Cell Proliferation and Differentiation

Nothing is more fundamental to living organisms than the ability to reproduce. Each time a human cell divides, it must duplicate its genome, a problem of biblical proportions. A single fertilized human egg contains 2.1 meters of DNA. An adult of about 75 kg (165 lb) consists of about 29 trillion cells containing a total of about 60 trillion meters of DNA, a distance equal to 400 times that of Earth to sun. Not only must the genome be duplicated trillions of times during human development, but it must be duplicated once and only once each time a cell divides (termed mitotic cell cycles). If we interfere with this process by artificially inducing cells to re-replicate their nuclear genome before cell division, the result is DNA damage, mitotic catastrophe, and programmed cell death (apoptosis). On rare occasions, specialized cells can duplicate their genome several times without undergoing cell division (termed endocycles), but when this occurs, it generally results in terminally differentiated polyploid cells, which are viable but no longer proliferate. However, as we age, the ability to regulate genome duplication diminishes, resulting in genome instability, which allows genetic alterations that can result in promiscuous cell division, better known as cancer.

Our research program focuses on three questions: the nature of the mechanisms that restrict genome duplication to once per cell division; how these mechanisms are circumvented to allow developmentally programmed induction of polyploidy in terminally differentiated cells; and how we can manipulate such mechanisms to destroy cancer cells selectively.

DHS (4,4-dihydroxy-trans-stilbene) suppresses DNA replication and tumor growth by inhibiting RRM2 (ribonucleotide reductase regulatory subunit M2) [Reference 1].

The DNA replication machinery is responsible for accurate and efficient duplication of the chromosome. Given that inhibition of DNA replication can lead to replication fork stalling, resulting in DNA damage and apoptotic death, inhibitors of DNA replication are commonly used in cancer chemotherapy. Ribonucleotide reductase (RNR) is the rate-limiting enzyme in the biosynthesis of deoxyribonucleoside triphosphates (dNTPs), which are essential for DNA replication and DNA-damage repair.
Gemcitabine, a nucleotide analog that inhibits RNR, has been used to treat various cancers. However, patients often develop resistance to the drug during treatment. Thus, the development of new drugs that inhibit RNR is needed. We identified a synthetic analog of resveratrol (3,5,4’-trihydroxy-trans-stilbene), termed DHS (4,4’-dihydroxy-trans-stilbene), which acts as a potent inhibitor of DNA replication. Molecular-docking analysis identified the RRM2 (ribonucleotide reductase regulatory subunit M2) of RNR as a direct target of DHS. At the molecular level, DHS induced cyclin F–mediated down-regulation of RRM2 by the proteasome. Thus, treatment of cells with DHS reduced RNR activity and consequently decreased synthesis of dNTPs with concomitant inhibition of DNA replication, arrest of cells at S-phase, DNA damage, and finally apoptosis. In mouse models of tumor xenografts, DHS was efficacious against pancreatic, ovarian, and colorectal cancer cells. Moreover, DHS overcame both gemcitabine resistance in pancreatic cancer and cisplatin resistance in ovarian cancer. Thus, DHS is a novel anticancer agent that targets RRM2 with therapeutic potential, either alone or in combination with other agents, to arrest cancer development.

A family of PIKFYVE inhibitors with therapeutic potential against autophagy-dependent cancer cells disrupt several events in lysosome homeostasis [Reference 2].

High-throughput screening identified five small-molecule chemical analogs (termed the WX8-family), that disrupted three events in lysosome homeostasis: (1) lysosome fission via tubulation without preventing homotypic lysosome fusion; (2) trafficking of molecules into lysosomes without altering lysosomal acidity; and (3) heterotypic fusion between lysosomes and autophagosomes. Remarkably, the compounds did not prevent homotypic fusion between lysosomes, despite the fact that homotypic fusion required some of the same machinery essential for heterotypic fusion. The effects varied 400–fold among WX8–family members, were time- and concentration-dependent, reversible, and resulted primarily from their ability to bind specifically to the PIKFYVE phosphoinositide kinase. The ability of the WX8 family to prevent lysosomes from participating in macroautophagy/autophagy suggested that they have therapeutic potential in treating autophagy-dependent diseases. In fact, the most potent WX8 family member was 100 times more lethal to ‘autophagy-addicted’ melanoma A375 cells than the lysosomal inhibitors hydroxychloroquine and chloroquine. In contrast, cells that were insensitive to hydroxychloroquine and chloroquine were also insensitive to the WX8 family. Therefore, the WX8 family of PIKFYVE inhibitors provides a basis for developing drugs that could selectively kill autophagy-dependent cancer cells, as well as for increasing the effectiveness of established anticancer therapies through combinatorial treatments.

The Cdk2–c-Myc–miR-571 axis regulates DNA replication and genomic stability by targeting geminin [Reference 3].

DNA re-replication leads to genomic instability and has been implicated in the pathology of a variety of human cancers. Eukaryotic DNA replication is tightly controlled to ensure that it occurs only once during each cell cycle. The nuclear protein Geminin is a critical component of this control: it prevents DNA re-replication from occurring during S, G2, and early M phases by preventing MCM helicases (essential for genomic DNA replication) from forming pre-replication complexes. Geminin is targeted for degradation by the anaphase-promoting complex (APC/C) from anaphase through G1 phase. However, accumulating evidence indicates that Geminin is downregulated in late S-phase by an unknown mechanism. We used a high-throughput screen to identify microRNAs that can induce excess DNA replication and found that the microRNA miR-571 could reduce the protein level of Geminin in late S-phase independently of the APC/C. Furthermore, miR-571 regulated efficient DNA replication and S-phase cell-cycle progression. Strikingly, the transcription factor c-Myc suppressed miR-
miR-571 expression by binding directly to the miR-571 promoter. At the beginning of S-phase, the cell-cycle regulator Cdk2 (cyclin-dependent kinase 2) phosphorylated c-Myc at serine 62, promoting its association with the miR-571 promoter region. Collectively, we identified miR-571 as the first miRNA that prevents aberrant DNA replication and the Cdk2–c-Myc–miR-571 axis as a new pathway for regulating DNA replication, the cell cycle, and genomic stability in cancer cells. The significance of such finding is that they identify a novel regulatory mechanism critical for maintaining genome integrity by regulating DNA replication and cell-cycle progression.

Cell-cycle arrest and apoptosis are not dependent on p53 prior to p53-dependent embryonic stem cell differentiation [Reference 4].

Previous efforts to determine whether or not the transcription factor and tumor suppressor protein p53 is required for DNA damage-induced apoptosis in pluripotent embryonic stem cells (ESCs) produced contradictory conclusions. To resolve the issue, p53+/+ and p53–/– ESCs derived by two different methods were used to quantify time-dependent changes in nuclear DNA content: annexin-V binding; cell permeabilization; and protein expression, modification, and localization. The results revealed that the chemotherapeutic drug doxorubicin (Adriamycin [ADR]) concentrations 10 to 40 times less than commonly used in previous studies induced the DNA damage–dependent G2–checkpoint and completed apoptosis within the same time frame, regardless of the presence or absence of p53, p21, or PUMA. Elevated ADR concentrations delayed initiation of apoptosis in p53–/– ESCs, but the rates of apoptosis remained equivalent. We obtained similar results by inducing apoptosis with either staurosporine inhibition of kinase activities or WX8 disruption of lysosome homeostasis. Differentiation of ESCs by deprivation of the cytokine LIF revealed p53–dependent formation of haploid cells, increased genomic stability, and suppression of the G2–checkpoint. Minimal induction of DNA damage now resulted in p53–facilitated apoptosis, but regulation of pluripotent gene expression remained p53–independent. Primary embryonic fibroblasts underwent p53–dependent total cell-cycle arrest (a prelude to cell senescence), and p53–independent apoptosis occurred in the presence of 10–fold higher levels of ADR, consistent with previous studies. Taken together, the results reveal that the many roles of p53 in cell-cycle regulation and apoptosis are first acquired during pluripotent stem cell differentiation.

Efficacy of a small-molecule inhibitor of the transcriptional cofactor PC4 in prevention and treatment of non–small cell lung cancer [Reference 5]

The human positive coactivator 4 (PC4) was originally identified as a multi-functional cofactor capable of mediating transcription activation by diverse gene- and tissue-specific activators. Recent studies suggest that PC4 might also function as a novel cancer biomarker and therapeutic target for various types of cancer. siRNA knockdown studies indicated that down-regulation of PC4 expression could inhibit tumorigenicity of A549 non-small cell lung cancer tumor model in nude mice. We showed that AG-1031, a small molecule identified by high-throughput screening, can inhibit the double-stranded DNA binding activity of PC4 more effectively than PC4's single-stranded DNA binding activity. AG-1031 also specifically inhibited PC4–dependent transcriptional activation in vitro using purified transcription factors. AG-1031 inhibited proliferation of several cultured cell lines derived from non–small cell lung cancers (NSCLC) and growth of tumors that formed from A549 cell xenografts in immuno-compromised mice. Moreover, pre-injection of AG-1031 in these mice not only reduced tumor size, but also prevented tumor formation in 20% of the animals. AG-1031–treated A549 cells and tumors from AG-1031–treated animals showed a significant reduction in the levels of both PC4 and of VEGFC, a key mediator of angiogenesis in cancer. On the other hand, the weight of all tested mice remained constant during animal trials. The results demonstrated that AG-1031 is be a potential therapy for PC4-positive NSCLC.
Publications


Collaborators

• Juan Bonifacino, PhD, Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD
• Marc Ferrer, PhD, Chemical Genomics Center, NCATS, Bethesda, MD
• Juan Marugan, PhD, Division of Pre-Clinical Innovation, NCATS, Bethesda, MD
• Zakir Ullah, PhD, Lahore University of Management Sciences, Lahore, Pakistan
• Wenge Zhu, PhD, George Washington University Medical School, Washington, DC

Contact

For more information, email melvin.depamphm@nih.gov or visit http://depamphilislab.nichd.nih.gov.
Mechanism and Regulation of Eukaryotic Protein Synthesis

We study the mechanism and regulation of protein synthesis, focusing on GTPases, protein kinases, translation factors, and mRNA features that control this fundamental cellular process. We use molecular-genetic and biochemical studies in yeast and human cells to dissect the structure-function properties of translation factors, elucidate mechanisms that control protein synthesis, and characterize how mutations in the protein synthesis apparatus cause human disease. Of special interest are the translation initiation factors eIF2, a GTPase that binds methionyl-tRNA to the ribosome, and eIF5B, a second GTPase that catalyzes ribosomal subunit joining in the final step of translation initiation. We also investigate stress-responsive protein kinases that phosphorylate eIF2alpha, as well as viral regulators of these kinases, and how cellular phosphatases are targeted to dephosphorylate eIF2alpha. We are characterizing eIF2gamma mutations that are associated with the MEHMO syndrome, a novel X-linked intellectual disability syndrome, and we are investigating the function of the translation factor eIF5A, with a focus on its ability to stimulate the peptidyl transferase activity of the ribosome and facilitate the reactivity of poor substrates such as proline. We are also examining the role of the hypusine modification on eIF5A and the role the factor plays in polyamine-regulated gene-specific translational control mechanisms, and we are characterizing metabolite control of translation via upstream Conserved Coding (uCC) regions in select mRNAs.

Analysis of eIF2gamma mutations that link intellectual disability with impaired translation initiation

Protein synthesis plays a critical role in learning and memory in model systems, and our studies have linked a human X-linked intellectual disability (XLID) syndrome to altered function of eIF2. In previous studies, we showed that the MEHMO syndrome (named based on the patient phenotypes: mental [intellectual] disability, epilepsy, hypogonadism and hypogenitalism, microcephaly, and obesity) is caused by mutations in the EIF2S3 gene, which encodes the gamma subunit of eIF2. Using genetic and biochemical techniques in yeast models of human MEHMO–syndrome mutations, we previously characterized several mutations that impair eIF2 function, disrupt eIF2 complex integrity, and alter the stringency of translation start-codon
selection. Our studies on the *EIF2S3–I259M* mutation, identified in patients with a severe form of the disease, revealed that the mutation impaired binding of the initiator methionyl-tRNA to eIF2 [Reference 1]. Over the past year, we generated yeast models of two additional *EIF2S3* mutations linked to the MEHMO syndrome [Reference 2]. These new mutations, which lie in the G domain of eIF2gamma, impaired yeast cell growth, altered translation and reduced stringency of translation start-site selection. Our collaborators in Germany linked the *EIF2S3* mutations with variable levels of motor delay, microcephaly, ID, epilepsy, central obesity and diabetes, thus broadening the genetic spectrum and clinical expressivity of MEHMO syndrome.

In additional studies from the last year, we characterized induced pluripotent stem (iPS) cells derived from a patient with the MEHMO syndrome [Reference 3]. In addition to a general reduction in protein synthesis, the cells constitutively induced the integrated stress response, with elevated expression of a translational regulatory response typically associated with eIF2alpha phosphorylation, and heightened expression of the transcriptional activators ATF4 and CHOP and the protein phosphatase-regulatory subunit GADD34. Under stress conditions, hyperactivation of the integrated stress response in the mutant iPS cells triggered apoptosis.

In addition, upon differentiation into neurons, the mutant cells exhibited reduced dendritic arborization. Our studies linking altered protein synthesis with intellectual disability are consistent with the critical role of protein synthesis in learning and memory in model systems. Based on our studies, we propose that the mutations in eIF2gamma impair the efficiency and fidelity of protein synthesis, and that such altered control of protein synthesis underlies the MEHMO syndrome. Addition of the drug ISRIB, an activator of the eIF2 guanine nucleotide exchange factor, rescued the cell-growth, translation, and neuronal-differentiation defects associated with the *EIF2S3* mutation, offering the possibility of therapeutic intervention for the MEHMO syndrome [Reference 3].

### Molecular analysis of the hypusine-containing protein eIF5A and polyamine control of protein synthesis

The translation factor eIF5A is the sole cellular protein containing the unusual amino acid hypusine (N^-{4}-amino-2-hydroxybutyl)lysine. In previous studies, we showed that eIF5A promotes translation elongation and that such activity depends on its hypusine modification. Moreover, using *in vivo* reporter assays, we showed that eIF5A in yeast, like its bacterial homolog EF-P, is especially critical for the synthesis of proteins containing runs of three or more consecutive proline residues. Moreover, using reconstituted *in vitro* translation assays, we showed that eIF5A was necessary for the synthesis of peptides containing polyproline sequences. In collaboration with Rachel Green, we reported that eIF5A functions globally to promote both translation elongation and termination. Moreover, using our *in vitro* reconstituted assay system, we showed that the structural rigidity of the amino acid proline contributes to its heightened requirement for eIF5A and that eIF5A could functionally substitute for polyamines to stimulate general protein synthesis [Reference 4].

The function of eIF5A to promote peptide bond formation is consistent with our structural studies with the X-ray crystallographer Marat Yusupov. The eIF5A was bound in the ribosome E site with the hypusine residue projecting toward the acceptor stem of the P-site tRNA. We propose that eIF5A and its hypusine residue function to reposition the acceptor arm of the P site to enhance reactivity towards either an aminoacyl-tRNA, for peptide bond formation, or a release factor, for translation termination.

In ongoing experiments, we are further investigating the hypusine modification on eIF5A. The modification is formed in two steps: first, an N^-{butyl}amine moiety from spermidine is transferred to a specific Lys side chain...
on eIF5A, whereupon hydroxylation on the added moiety completes the formation of hypusine. In contrast to the essential deoxyhypusine synthase, which catalyzes the first step in hypusine formation, the LIA1 gene encoding the hydroxylase is non-essential in yeast. We identified mutations in eIF5A that cause synthetic growth defects in cells lacking the hydroxylase. Interestingly, the mutations map to the ribosome-binding face of eIF5A. Our results are consistent with the notion that the hydroxyl modification helps bind and position eIF5A and its hypusine residue to effectively promote the reactivity of the peptidyl-tRNA on the ribosome.

Recently, we linked eIF5A to the regulation of polyamine metabolism in mammalian cells [Reference 5]. The enzyme ornithine decarboxylase (ODC) catalyzes the first step in polyamine synthesis. ODC is regulated by a protein called antizyme, which, in turn, is regulated by another protein called antizyme inhibitor (AZIN1). The synthesis of AZIN1 is inhibited by polyamines, and an element in the leader of the AZIN1 mRNA is critical for the regulation. The element resembles an upstream open reading frame (uORF); however, we refer to it as an upstream Conserved Coding (uCC) region, because the element lacks an AUG start codon and initiates at a near-cognate codon instead. Whereas translation initiation is typically restricted to AUG codons, and scanning eukaryotic ribosomes inefficiently recognize near-cognate start codons, we found that high polyamine levels enhance translation initiation from the near-cognate start site of the uCC. Remarkably, the polyamine induction of uCC translation depends on the sequence of encoded polypeptide, including a highly conserved Pro-Pro-Trp (PPW) motif. Ribosome profiling revealed polyamine-dependent pausing of elongating ribosomes on the PPW motif in the uCC, and mutation of the PPW motif impaired initiation at the near-cognate AUU start codon of the uCC and abolished polyamine control, leading to constitutive high-level expression of AZIN1. We proposed that scanning ribosomes typically bypass the near-cognate start codon of the uCC without initiating and then translate AZIN1. However, occasionally a ribosome will initiate translation at the uCC start codon. Under conditions of high polyamine levels, the elongating ribosomes pause on the PPW motif. The paused ribosome serves as a roadblock to subsequent scanning ribosomes that bypass the near-cognate start codon. The resulting queue of scanning ribosomes behind the paused elongating ribosome positions a ribosome near the start site of the uCC, providing greater opportunity for initiation at the weak start site. Consistent with this queuing model, we found that impairing ribosome loading, and thus queue formation, reduced uCC translation and derepressed AZIN1 synthesis.

In further studies on the AZIN1 regulatory mechanism, we identified eIF5A as a sensor and effector for polyamine control of uCC translation. Using reconstituted in vitro translation assays, we found that synthesis of a PPW peptide, like translation of polyproline sequences, requires eIF5A. Moreover, the ability of eIF5A to stimulate PPW synthesis was inhibited by polyamines and could be rescued by increasing eIF5A levels. We propose that polyamines interfere with eIF5A binding on the ribosome and that inhibition of eIF5A serves as the trigger to cause the ribosome pause that governs uCC translation. Taken together, our studies showed that eIF5A functions generally in protein synthesis and that modulation of eIF5A function by polyamines can be exploited to regulate specific mRNA translation [Reference 5]. We are now exploring the possibility that polyamine regulation of eIF5A underlies translational control of mRNAs encoding other enzymes and regulators of polyamine biosynthesis.

Translational control by metabolite-sensing nascent peptides

In ongoing studies, we searched for additional mRNAs containing potential uCCs. One such candidate was identified in plants in the mRNA encoding GDP-L-galactose phosphorylase (GGP), a control enzyme in the vitamin C biosynthetic pathway. Using reporter assays in mammalian cells and in vitro using rabbit
reticulocyte lysates, we revealed that a uORF–like element in the GGP mRNA is a uCC. We propose that interaction of vitamin C with the GGP uCC nascent peptide in the ribosome exit tunnel causes the ribosome to pause and that queuing of subsequent scanning ribosomes results in increased initiation on the uCC and prevents ribosome access to the GGP ORF. We hypothesize that the mechanism by which a paused elongating ribosome promotes initiation at an upstream weak start site via ribosome queuing may underlie the control of translation of other mRNAs, especially those whose translation is derepressed by conditions that impair ribosome loading.

**Analysis of the role of eEF2 and its diphthamide modification in translation elongation and CrPV IRES translation**

We are also studying the translation elongation factor eEF2. Like its bacterial ortholog EF-G, eEF2 promotes translocation of tRNAs and mRNA from the A site to the P site on the ribosome, following peptide bond formation. In all eukaryotes and archaea, a conserved histidine residue at the tip of eEF2 is post-translationally modified to diphthamide through the action of seven non-essential proteins. The function of diphthamide and the rationale for its evolutionary conservation are not well understood. The name diphthamide is derived from diphtheria, a disease of the nose and throat caused by the bacterium *Corynebacterium diphtheriae*. Infections with *C. diphtheriae* can lead to respiratory distress and even death; however, an effective vaccine is available. The bacterium expresses a toxin that ADP–ribosylates the diphthamide residue, leading to inactivation of eEF2. Of note, several additional bacterial pathogens, including *Pseudomonas aeruginosa* and *Vibrio cholerae*, express distinct toxins that likewise modify the diphthamide residue and inactivate eEF2.

To gain insights into the role of eEF2 and diphthamide, we screened for mutations that sensitize eEF2 to loss of the diphthamide modification and are currently characterizing the mutants. In previous studies, we showed that loss of diphthamide does not affect *in vitro* peptide synthesis directed by the canonical initiation pathway; however, synthesis of the same peptide directed by the novel cricket paralysis virus (CrPV) internal ribosome entry site (IRES), which bypasses canonical translation initiation and instead requires novel eEF2–directed pseudo-translocation reactions prior to peptide bond formation, is sensitive to the loss of diphthamide. Based on a cryo-electron microscopy structure of eEF2 and the CrPV–IRES bound to the yeast 80S ribosome obtained by our collaborators in Venki Ramakrishnan’s lab, we hypothesize that diphthamide has at least two functions: first, to disrupt the decoding interactions of rRNA with the codon-anticodon duplex in the ribosomal A site; and second, to help chaperone the codon-anticodon interaction as the A-site tRNA is translocated to the P site.

In ongoing studies, we are further exploring the role of diphthamide in promoting the accuracy and efficiency of translation elongation. Our preliminary data indicate that loss of diphthamide impairs the fidelity of translation leading to increased levels of ribosome frameshifting. Accordingly, we propose that diphthamide, despite its non-essential nature in yeast, has been conserved throughout evolution to maintain the fidelity of translation elongation and block spurious frameshifting events that would limit the production of the proteins.

**Publications**

2. Kotzaeridou U, Young-Baird SK, Suckow V, Thornburg AG, Wagner M, Harting I, Christ S, Strom T, Dever TE, Kalscheuer VM. Novel pathogenic EIF2S3 missense variants causing clinically variable MEHMO syndrome


Collaborators

• John Atkins, PhD, University College Cork, Cork, Ireland
• Israel Fernandez, PhD, Columbia University, New York, NY
• Adam Geballe, MD, The Fred Hutchinson Cancer Research Center, Seattle, WA
• Rachel Green, PhD, The Johns Hopkins University School of Medicine, Baltimore, MD
• Vera Kalscheuer, PhD, Max Planck Institute for Molecular Genetics, Berlin, Germany
• Terri Goss Kinzy, PhD, Western Michigan University, Kalamazoo, MI
• Eric Klann, PhD, New York University, New York, NY
• Stefan Leibau, MD, Eberhard Karls Universität Tübingen, Tübingen, Germany
• Joseph Puglisi, PhD, Stanford University, Palo Alto, CA
• Venkatraman Ramakrishnan, PhD, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom
• Matthew Sachs, PhD, Texas A&M University, College Station, TX
• Hiroaki Suga, PhD, University of Tokyo, Tokyo, Japan
• Marat Yusupov, PhD, Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Université de Strasbourg, Strasbourg, France

Contact

For more information, email thomas.dever@nih.gov or visit http://deverlab.nih.gov.
Regulation and Functional Relevance of Retrograde Transport in Axons

Cytoplasmic dynein is the single motor responsible for microtubule minus-end (cell body-directed) axonal transport. The importance of this retrograde motor to neural health is apparent: mutations in the dynein-dynactin complex are the cause of subtypes of neurological disease. Additionally, the abnormal localization of dynein-dependent cargos is associated with disease states. Although dynein function appears essential for neural health, the mechanisms that govern precise cargo movement by the motor and how that impacts neural circuit structure and function are almost completely unknown.

One dynein cargo of critical importance to axonal physiology is the mitochondrion. Axons depend on the proper localization of mitochondria for sufficient ATP synthesis and calcium buffering, as well as for lesser known functions such as production of metabolites, synthesis of signaling molecules, and iron homeostasis. Abnormalities in mitochondrial localization are correlated with neurological disease; however, whether there is a causal relationship between organelle movement and disease has been difficult to determine. Our work on mitochondrial transport and function in relation to neural circuit activity aims at an understanding of the regulation of retrograde axonal transport and how it impacts the nervous system.

Using the novel tools that we developed, namely, forward and reverse genetics, advanced imaging of intracellular phenomena in vivo, and analyses of neural circuit function, we interrogate the molecular regulation of cargo-specific retrograde transport in axons and determine the role that movement of each of these cargos plays in the formation and maintenance of functional neural circuits.

Regulation and functional significance of retrograde axonal transport
MITOCHONDRIAL RETROGRADE TRANSPORT MECHANISMS AND FUNCTION
Mitochondrial transport is necessary to properly position the organelle in axons. Correct mitochondrial localization in axons is critical for energy production near sites of high metabolic demand, for calcium homeostasis to regulate neuronal activity, and to regulate axonal branching in certain contexts. Various model systems have revealed several mediators of mitochondrial movement. Anterograde
Mitochondria accumulate in actr10–mutant axon terminals as a result of failed retrograde transport.

A. 5–dpf (days post-fertilization) TgBAC(neurod:egfp) transgenic zebrafish larva expressing GFP in all neurons. Mitochondrially localized TagRFP mosaically expressed.

A’. Posterior lateral line (pLL) ganglion with one neuron expressing TagRFP in mitochondria (circled). Arrowheads point to mitochondria in the single labeled axon.


A``` Arrowheads point to mitochondria in the axon terminal of this system.

B. Mitochondrial accumulation in actr10–mutant axon terminals (arrows).

C. Retrograde mitochondrial transport is disrupted in this mutant strain.

mitochondrial transport requires the Kinesin-1 molecular motor in association with several other proteins, including Miro and Milton. Interestingly, loss of Miro or Milton, the two best characterized adaptors for mitochondrial transport, eliminates all mitochondrial movement. Therefore, how this organelle is selectively transported in the retrograde direction is still unclear.

In a forward-genetic screen, we identified a novel zebrafish mutant strain with selective loss of retrograde mitochondrial transport. The causative mutation in this line results in loss of Actr10, a protein known to associate with the dynein motor. In actr10 mutants, anterograde mitochondrial transport is intact, but retrograde mitochondrial transport frequency is dramatically reduced, leading to accumulation of mitochondria in actr10–mutant axon terminals (Figure 1). Using mitochondrial fractionation, we demonstrated that the loss of retrograde transport frequency is attributable to loss of mitochondria-dynein interaction in the absence of Actr10. We are currently using the actr10 mutant as a tool to determine the impact of mitochondrial retrograde transport disruption on mitochondrial health and function. Additionally, we are working to address the normal rate and function of mitochondrial turnover in axons. For this, we made a stable transgenic zebrafish line expressing the photoconvertible protein mEos in the inner mitochondrial membrane space. Somewhat surprisingly, just three hours after photoconversion, the old (converted) mitochondria had evacuated from the axon terminal and new (unconverted) mitochondria had taken their place (Figure 2), which is in contrast to the predictions based on shorter-term imaging conducted within the order of minutes in cultured neurons. The findings demonstrate the power of an entirely in vivo system for observing cellular phenomena on a time-scale relevant to the organism.
FIGURE 2. Mitochondria turn over in axon terminals within hours.

A. Photoconversion of mEos in the inner mitochondrial membrane space labels axon terminal mitochondria (magenta in A'). Twenty-four hours after conversion, no converted (old) mitochondria remain.

B. Quantification of old (red) to new (green) mitochondria before (pre), just after (post), and 24hr after (24hr post) conversion.

C. Time-lapse imaging revealed that mitochondrial turnover occurs about 3hrs after conversion in 4–5 dpf larvae.

In collaboration with the Kindt lab, we are investigating the impact of mitochondrial accumulation on the health and function of the axon. The work will define the molecular mechanisms of retrograde mitochondrial transport and provide insights into how loss of this specific cellular activity impacts the organelle, the cell, and the neural circuit in vivo.

MICROTUBULES, DYNEIN STABILITY, AND NUDC

Our forward-genetic screen yielded several other mutant lines, including one with a loss-of-function mutation in the dynein-associated protein NudC. In mammalian systems, loss of NudC results in failed mitosis and abnormal neuronal migration. The function of NudC in such phenomena is still under investigation. During cell division, loss of NudC results in abnormal microtubule orientation and failure of kinetochore complex formation. Studies on migrating neurons indicated that loss of NudC impacts the stability of the dynein motor protein complex. Whether NudC has similar or disparate functions during different stages of development in neurons is thus still highly debated. The NudC mutant identified in our screen will allow us to address the role of NudC in mature axons in vivo.
NudC mutants do not phenocopy dynein or dynactin loss-of-function mutants, with only axon branchpoint swellings in distal axons and no signs of either axonal or retinal degeneration. In collaboration with the Petralia lab, we used transmission electron microscopy to reveal that loss of NudC results in multilamellar body formation in the swellings (Figure 3). In vivo analyses of axonal transport of autophagosome- and endosome-related cargos revealed that most show disruption of their axonal transport; however, the disruptions are not consistent, leading to the question as to whether the structure or arrangement of the microtubule cytoskeleton is effected in this line. Currently, we are using pharmacology, live imaging of microtubule dynamics, and expansion microscopy to gain insight into the structure, stability, and dynamics of the microtubule cytoskeleton in this line in order to understand the function of NudC in a mature axon. The work will ultimately permit us to determine whether NudC serves a conserved function in developing neurons, which, when disrupted, leads to varying phenotypes, depending on developmental state.

Screening for novel regulators of cargo-specific retrograde axonal transport

Several pieces of evidence substantiate the importance of retrograde axonal transport for axon health and function. First, mutations in dynein and dynein-associated proteins are correlated with neurological disease. Second, retrograde transport of signaling endosomes is essential for the extension and maintenance of long axons. Third, abnormal localization of various cargoes, including mitochondria, correlates with neuronal disease. Despite the importance of the process, little is known about how various cargoes attach to and are then transported by the retrograde motor protein complex. We are using forward and reverse genetics in zebrafish to identify mediators of dynein-specific retrograde transport in axons.

Using a double transgenic line in a three-generation, forward-genetic screen, we are identifying recessive mutant strains with axon abnormalities characteristic of disruptions in retrograde axonal transport. Our previous work and that of others showed that the phenotypes include axon terminal swellings such as those observed in actr10 mutants (Figure 3). After identifying the strain, we use RNA-sequencing approaches to identify the causal mutation. To date, we have identified three lines, which all have mutations in dynein-associated proteins. At present, we are using the immuno-labeling approaches and in vivo imaging techniques.
that we previously developed to determine whether the strains have deficits in the retrograde transport of specific cargoes. We can use the mutant strains to first identify the proteins involved in the retrograde transport of particular cargoes and then as tools to determine how the specific disruptions affect the function of the axon.

**Publications**


**Collaborators**

- Tory Herman, PhD, *University of Oregon*, Eugene, OR
- Katie Kindt, PhD, *Section on Sensory Cell Development and Function, NIDCD, Bethesda, MD*
- Ronald Petralia, PhD, *Advanced Imaging Core, NIDCD, Bethesda, MD*
- Michael Ward, MD, PhD, *Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda, MD*

**Contact**

For more information, email [katie.drerup@nih.gov](mailto:katie.drerup@nih.gov) or visit [http://dreruplab.nichd.nih.gov](http://dreruplab.nichd.nih.gov).
SECTION ON MOLECULAR ENDOCRINOLOGY

Receptors and Actions of Peptide Hormones and Regulatory Proteins in Endocrine Mechanisms

The Section investigates the molecular basis of peptide hormones and their receptors in the control of gonadal function. Studies encompass the structure and transcriptional regulation of the luteinizing-hormone (LH) and prolactin receptors. We study the regulatory mechanism(s) involved in the progression of spermatogenesis and the control of Leydig cell function. We investigate novel gonadotropin-regulated genes relevant to the progression of testicular gametogenesis, Leydig cell function, and other endocrine processes. Our studies concentrate on the function and regulation of the gonadotropin-regulated testicular RNA helicase (GRTH/DDX25), a member of the DEAD-box family of RNA helicases discovered, cloned and characterized in our laboratory, which is essential for the progress of spermatogenesis. The various functions of GRTH/DDX25 provide fertile ground for the development of a non-hormonal male contraceptive.

Gonadotropin–regulated testicular RNA helicase

GRTH/DDX25 is present in Leydig cells (LC), meiotic (pachytene spermatocytes), and haploid germ cells (round and elongated spermatids) [Reference 1]. Males lacking GRTH are sterile owing to azoospermia, which results from failure of round spermatids (RS) to elongate. We demonstrated its participation in the nuclear export/transport of specific mRNAs, the structural integrity of the chromatoid body (CB), and in storage/processing of relevant mRNAs and their transit to and association with actively translating polyribosomes, where GRTH may regulate translational events. GRTH is the only member of the DEAD-box family regulated by hormones. GRTH transcription is stimulated in LCs by LH/cAMP through direct actions of androgen (A) and the A receptor (AR) (autocrine), and indirectly in germ cells (paracrine) through the AR in Sertoli cells. The upstream region of the GRTH gene directs its expression in germ cells and downstream in the LC. Through these regions, A/AR exerts their direct (endogenous) regulation of the GRTH gene in LC and indirectly in germ cells. Functional binding sites for germ-cell nuclear factor (GCNF) present in RS and spermatocytes (SP) and its regulation by A/AR were identified in the distal region of the GRTH gene, operative selectively in RS. Current knowledge indicates actions of A on GCNF cell-specific regulation of GRTH expression in germ cells (RS). Also, we previously established

Maria L. Dufau, MD, PhD, Head, Section on Molecular Endocrinology
Raghuveer Kavarthapu, PhD, Staff Fellow
Muruganath Kumar Raju, PhD, Postdoctoral Fellow
Rajakumar Anbazhagan, PhD, Visiting Fellow
that GRTH exerts negative autocrine regulation of GCNF, linking A actions to germ cells through GCNF as an A-regulated transfactor that controls transcription/expression of GRTH, providing a connection between androgen action and two relevant germ-cell genes (GRTH and GCNF), which are essential for the progress of spermatogenesis, and establishing their regulatory interrelationship.

Our early studies revealed that missense mutation of R to H at amino acid 242 of GRTH, found in 5.8% of patients with complete loss of sperm, causes loss of the 61 kDa phospho-species (pGRTH), with preservation of the 52 kDa nonphospho form. The finding provided an avenue through which to elucidate the function of pGRTH in spermatogenesis. We generated humanized mutant GRTH knock-in (KI) mice [Reference 2]. The mice are sterile, with reduced testicular size and lack of sperm (arrest at step 8 of RS), and demonstrate complete loss of the pGRTH species but with preservation of the nuclear 52 kDa form. The mouse model permits the study of the biological/biochemical functions of the cytoplasmic pGRTH. In KI mice, the nuclear export transport and functions of GRTH are preserved (i.e., mRNA export, miRNA regulation), while the cytoplasmic functions, including shuttling of messages, storage in the CB, and translational events all requiring pGRTH, are absent. We
observed marked reduction in the size of the CB in RS and lack of pGRTH in the CB. Germ cell apoptosis was present in pachytene spermatocytes (PS) and RS. In contrast to knockout (KO) mice, KI mice showed no changes in miRNA biosynthesis, which excludes participation of pGRTH as transcriptional regulator of the microprocessor complex (the RNase III Drosha and the RNA–binding protein DCGR), which affects primiRNAs formation, but indicates the participation of non-phospho GRTH in these processes. In KI mice, there is loss of chromatin remodeling and of related proteins, including TP2, PRM2, and TSSK6. Significant decreases in their mRNA and half-lives indicate that their association with pGRTH in the cytoplasm protects such mRNAs from degradation. Also, our previous work showed that pGRTH stimulates TP2 translation in a 3’ UTR–dependent manner.

In recent studies, we elucidated the GRTH phospho-site at a threonine (Tr239), which is structurally adjacent to the mutant site found in patients (R242H) [Reference 3]. Molecular modelling of the phospho-site, based on the RecA domain 1 of the DDX19 (an RNA helicase) crystal structure, pointed to the amino acids that formed the GRTH/PKA interface, solvent accessibility, and H-bonding. In addition to the core residues T239 and R242, these include amino acids E165, K240, and D237. The relevance of these residues was demonstrated by single or double mutations that caused reduction in or abolition of the p-GRTH at Tr239. pGRTH is the cytoplasmic form, essential for the progress of spermatogenesis beyond step 8 of round spermatids, and consequent lack of sperm formation [Reference 4]. The deleterious effects on GRTH phosphorylation caused by the mutations did not result from changes of protein kinase A alpha (PKAα) catalytic binding affinity but rather from consequential structural changes that can affect PKA catalytic efficiency. Studies based on the abolition of the phospho-form provide the basis for drug design and virtual and throughput screening for discovery of a reversible chemical inhibitor for use as male contraceptive. During the past year, we determined that cyclic peptides that fit the shallow pocket of GRTH/protein kinase A are preferred compounds to block GRTH phosphorylation and amenable for use in the development of oral non-hormonal male contraceptives.

Work on transcriptome analysis of mice germ cells using RNA-Seq provided further insights regarding linking pGRTH to histone ubiquitination and acetylation, essential for chromatin compaction and spermatid development during spermiogenesis [Reference 4] (Figure 1). We also initiated studies on the role of phosphorylated GRTH in the storage of messages in the CB, an organelle present in germ cells and found to be a key site for the storage of mRNAs of relevance for the progress of spermatogenesis.

**Publications**

Collaborators

• Sergio A. Hassan, PhD, Center for Molecular Modeling, CIT, NIH, Bethesda, MD

Contact

For more information, email dufau@mail.nih.gov or visit https://irp.nih.gov/pi/maria-dufau.
Animals consist of a collection of cells with diverse shapes, structures, and functions, a diversity that is rebuilt from scratch by every embryo. Understanding the genetic programs that direct the process is the central mystery of developmental and regenerative biology. We are interested in how decisions about what cell type to adopt are controlled, and what genetic programs direct the morphological and functional specialization of different cells.

The single-cell revolution in developmental biology has given us new access and new tools to address these questions. I previously developed high temporal-resolution single-cell RNA sequencing approaches to identify transcriptional trajectories, i.e., the ‘highways’ or most likely paths through gene expression that cells take during development. From such data, we were able to identify the sequence of genes expressed by individual cell types during early development, which provides insight into the genetic programs that regulate cells’ choice of cell type and then their downstream functional transformations at a wider breadth than was previously achievable.

Work in the lab focuses on more deeply exploring such processes, using the approaches we developed. Our lab combines single-cell genomics, imaging, genetic, and classical embryological approaches to investigate the genetic control of cell specification and differentiation during vertebrate embryogenesis. We focus on zebrafish embryos as a model system to study these questions, because among vertebrates, they are easy to culture, image, and manipulate, both embryologically and genetically.

Genetic underpinnings of cell differentiation
Once a cell has been specified, it must acquire the particular morphology and functionality of its cell type through the process of differentiation, a process that is driven by cell type–specific expression of differentiation genes and often results in dramatic changes in basic cell-biological processes. We aim to identify those genes that drive differentiation and understand their regulation. To do this, we will generate a single-cell RNAseq atlas spanning a larger timespan of zebrafish embryonic development, find the cascade of genes expressed during differentiation in every cell type, determine their membership in functional gene modules (groups of genes that
work together), and associate them with that cell type’s cell-biological transformations during differentiation. The studies will permit comparisons of differentiation-gene deployment across cell types to understand the reuse of differentiation programs during development.

Consequences of heterogeneous developmental trajectories

Distinct cell types can arise through many developmental trajectories or developmental histories. We and others have observed refinement at the boundaries between groups of cells specified to become different tissues; at such boundaries, some cells switch from one specification state to another. We use the axial mesoderm as a model and seek to understand: (1) what drives cell-type switching; (2) what are the long-term consequences for a cell that switched; and (3) what mechanisms assist in successful switching.

Effect of environmental insults on developmental choices

During early embryogenesis, a field of equipotent cells are instructed to initiate different gene-expression programs by external developmental signals and cell-intrinsic cues. We recently observed that cells that experience DNA damage in early zebrafish embryos initiate an unusual transcriptional response during a very limited window in development. Moreover, most damaged cells are not eliminated but appear to be excluded from contributing to some tissues in the animal, which suggests that responding to DNA damage may affect cells’ choices during development and which raises a question of how that occurs. We are investigating: (1) the fate of cells in early development that experience DNA damage; (2) the role this unusual transcriptional response plays; and (3) what drives the bias in damaged cells’ future cell type.
Publications


Collaborators

- James Gagnon, PhD, *The University of Utah, Salt Lake City, UT*
- Celina Juliano, PhD, *University of California, Davis, CA*

Contact

For more information, email *jeffrey.farrell@nih.gov.*
Healthy brain and cognitive development in children is central to the mission of the NICHD. Our research is concerned with understanding the molecular and cellular mechanisms by which functional activity in the brain regulates development of the nervous system during late stages of fetal development and early postnatal life. In addition to synaptic plasticity, we are interested in novel mechanisms of activity-dependent nervous system plasticity that are particularly relevant to the period of childhood, including the involvement of glia (non-neuronal brain cells). Our work has three main areas of emphasis: myelination and neuron-glia interactions; cellular mechanisms of learning; and gene regulation by neuronal firing.

Traditionally, the field of activity-dependent nervous system development has focused on synapses, and we continue to explore synaptic plasticity. However, our research is also advancing our understanding of how glia sense neural impulse activity and how activity-dependent regulation of glia contributes to development, plasticity, and the cellular mechanisms of learning. A major emphasis of our current research is to understand how myelin (white matter in the brain) is regulated by functional activity. By changing conduction velocity, activity-dependent myelination may be a non-synaptic form of plasticity, regulating nervous system function by optimizing the speed and synchrony of information transmission through neural networks. Our studies have identified several cellular and molecular mechanisms for activity-dependent myelination, and the findings have important implications for normal brain development, learning, cognition, and psychiatric disorders. Our research shows that myelination of axons by glia (oligodendrocytes in the CNS and Schwann cells in the peripheral nervous system [PNS]) is regulated by impulse activity, and we have identified several molecular mechanisms that control proliferation and differentiation of myelinating glia and myelination. Most recently, we determined that myelin thickness can be adjusted through a treadmilling process that adds and removes layers of myelin from the sheath to adjust conduction velocity and improve functional performance by optimizing spike-time arrival at synapses. The findings provide evidence for a new form of nervous system plasticity and learning that would be particularly important in child development, but which also operates in adulthood, thereby improving function based on experience.
Learning is perhaps the most important function of childhood. Our research is determining the molecular mechanisms that convert short-term memory into long-term memory. If functional experiences produce lasting effects on brain development and plasticity, specific genes must be regulated by specific patterns of impulse firing. We are determining how various patterns of neural impulses regulate specific genes controlling development and plasticity of neurons and glia, and how synaptic strength is modified in the hippocampus.

**Nervous system plasticity by activity-dependent myelination**

Myelin, the multilayered membrane of insulation wrapped around nerve fibers (axons) by glial cells, is essential for proper neural impulse transmission and nervous system function. Myelination is an essential part of brain development, but the processes controlling myelination of appropriate axons are not well understood. Myelination begins in late fetal life and continues throughout childhood and adolescence, but myelination of some brain regions is not complete until an individual's early twenties.

Traditionally myelin has been viewed in terms of conduction failure after damage (for example in multiple sclerosis), but we are exploring how changes in myelin driven by functional activity affect the timing of neural-impulse arrival at synaptic relay points, which is critical for information processing and synaptic activity. In addition, the frequency, phase, and amplitude coupling of oscillations in the brain (brainwaves) requires appropriate impulse conduction velocity, which is influenced by myelination. Many neurological and psychological dysfunctions can develop when optimal neural synchrony of spike-time arrival and neural oscillations are disturbed; for example, in schizophrenia, epilepsy, dyslexia, and autism.
Our research shows that, to activate receptors on myelinating glia as well as on astrocytes and other cells, neurotransmitters are released not only at synapses but also along axons firing action potentials. The recipient cells in turn release growth factors, cytokines, and other molecules that regulate myelination, proliferation, and development of myelinating glia.

**INDUCTION OF MYELINATION BY ACTION POTENTIALS**

In addition to establishing the effects of impulse activity on proliferation and development of myelinating glia, we determined that release of the neurotransmitter glutamate from vesicles along axons triggers the initial events in myelin induction, including stimulating the formation of cholesterol-rich signaling domains between oligodendrocytes and axons and increasing the local synthesis of myelin basic protein, the major protein in the myelin sheath, through Fyn kinase–dependent signaling. We showed that, through this axon-oligodendrocyte signaling mechanism, electrically active axons become preferentially myelinated by a factor of 8 to 1 over electrically inactive axons, thus regulating myelination of axons and neural circuit function according to functional experience, which would be particularly important in the adolescent brain, for example, where environmental experience during sensitive periods can have long-lasting effects on neural circuit development and behavior. The findings are also relevant to such demyelinating disorders as multiple sclerosis and to re-myelination after axon injury.

**MODIFICATION OF MYELIN STRUCTURE AND CONDUCTION VELOCITY BY ASTROCYTES**

Given that optimal neural-circuit function and synaptic plasticity require the proper impulse transmission speed through all axons to induce spike timing–dependent plasticity and to sustain oscillations at appropriate frequencies, mechanisms that determine and modify conduction time through axons could provide a non-synaptic mechanism of neural circuit plasticity. Conduction velocity in myelinated axons depends on the thickness of the myelin sheath and the morphology of the electrogenic nodes of Ranvier (gaps in the myelin sheath) along axons. Our research showed that myelination of unmyelinated axons and the thickness of the myelin sheath can be increased in response to neural activity and environmental experience. Prior to our research, myelin structure was believed to be static, and there was no known mechanism that could reduce the thickness of the mature myelin sheath (except in the context of pathology). However, a mechanism would be necessary to reduce conduction velocity to achieve optimal spike-time arrival from inputs that arrive at relay points in neural networks too soon.

Our research shows that myelin thickness and nodal gap length are reversibly altered by astrocytes, glial cells that contact nodes of Ranvier, and that this alters the speed of impulse transmission and neural network function. Myelin is attached to the axon by intercellular junctions adjacent to the nodes of Ranvier. We found that one of these cell-adhesion molecules (neurofascin 155) has a binding site for the proteolytic enzyme thrombin, which is secreted by neurons and enters the brain from the vascular system. We found that thrombin-dependent cleavage of neurofascin 155 severs the tether between the axon and myelin, allowing the latter to detach and rendering the myelin sheath thinner. The process is inhibited by vesicular release of thrombin protease inhibitors from perinodal astrocytes. Previously, it was unknown how the myelin sheath could be thinned, and the functions of perinodal astrocytes were not well understood. Our findings uncover a new form of nervous system plasticity in which myelin structure and conduction velocity are adjusted by astrocytes. The thrombin-dependent cleavage of neurofascin 155 may also have relevance to myelin disruption and repair.
FIGURE 2. Treadmilling model for plasticity of the myelin sheath

The speed of neural impulse transmission is altered by changes in myelin structure. The thickness of the myelin sheath in central nervous system axons is determined by two opposing processes: one (A) that adds additional wraps of myelin to the axon, and the other (B) that removes the outer layer, thereby increasing and decreasing impulse conduction velocity, respectively. New layers of myelin are added beneath the overlaying layers by expansion of the inner tongue of myelin. Myelin is attached to the axon at the paranodal region flanking the node of Ranvier via septate-junctions, comprised of neurofascin 155 on myelin interacting with Contactin1-Caspr1 complex on the axon. Cleavage of neurofascin 155 by thrombin (red) can break this interaction, resulting in detachment of the outer paranodal loop from the axon, and withdrawal of the outer layer of myelin, which increases nodal gap length and reduces myelin sheath thickness; both effects slow conduction velocity. Perinodal astrocytes at the nodes of Ranvier regulate the process by secreting thrombin inhibitors (green triangle), such as Protease Nexin1. The treadmilling process helps achieve optimal conduction velocity in individual axons [Reference 4].

GULF WAR ILLNESS

After decades of research, there is still no understanding of how a large group of Gulf War veterans became chronically ill with Gulf War Illness. It is believed that exposure to low levels of sarin nerve gas and combinations of organophosphate insecticides, which impair synaptic function, may be responsible. Our discovery that glutamatergic transmission between axons and oligodendrocytes triggers myelination led us to propose that impairments in myelination caused by disrupted neurotransmission from axons to oligodendrocytes may be an underlying cause of Gulf War Illness. Our research shows that proliferation and development of oligodendrocytes is affected in an animal model of Gulf War illness and in cultures of oligodendrocytes exposed to agents such as sarin nerve gas (acetylcholinesterase inhibitors). Perturbations by these agents of axon-glial interactions, which take place through acetylcholine signaling, could have long-lasting consequences in neural network functions underlying many of the symptoms associated with Gulf War Illness, including among others difficulties with working memory, mental focus, and chronic pain. Organophosphate pesticides operate in a similar manner, and exposures to pesticide contamination, especially in childhood, would impair normal development of oligodendrocytes and myelin formation, contributing to cognitive and psychological dysfunctions.
Regulation of gene expression by action potential firing patterns

All information in the nervous system is encoded in the temporal pattern of neural impulse firing. Given that long-lasting changes in the nervous system require regulated gene expression, appropriate patterns of neural impulse firing driving neural plasticity must control transcription of specific genes, a fundamental question central to the processes of experience-dependent plasticity during development and learning. However, little is known about how neural firing patterns regulate gene expression. Our experiments are revealing the intracellular signaling and gene-regulatory networks that respond selectively to appropriate temporal patterns of action-potential firing to generate adaptive responses.

To determine how gene expression in neurons and glia is regulated by impulse firing, we stimulate nerve cells to fire impulses in differing patterns by optogenetics and by delivering electrical stimulation through platinum electrodes in specially designed cell-culture dishes. Live-cell calcium imaging shows that temporal aspects of intracellular calcium signaling are particularly important in regulating gene expression according to neural-impulse firing patterns in normal and pathological conditions. After stimulation, we measured mRNA and protein expression by gene microarrays, quantitative RT-PCR (reverse transcriptase–polymerase chain reaction), RNA-seq (RNA sequencing), Western blot, and immunocytochemistry. The results confirm our hypothesis that precise patterns of impulse activity can increase or reduce expression of specific genes in neurons and glia. Moreover, our research shows that regulation of gene expression in neurons by specific temporal patterns of impulse activity is not a property of special genes; in general, the neuronal transcriptome is highly regulated by the pattern of membrane depolarization, with hundreds of genes differentially regulated by the temporal code of neuronal firing.

We are also pioneering new methods of transcriptional analysis in neurons. The standard approach to analyzing gene expression is by measuring the abundance of tens of thousands specific gene transcripts in cells by microarray or RNA-seq, as described above, but this approach fails to capture the unique feature of transcriptional regulation in neurons. In contrast to other cells responding to external signals that may drive cells to a steady-state equilibrium, transcriptional networks in neurons are continually modulated dynamically by temporally varying action-potential firing frequencies and burst patterns, together with synchrony and phase relationships among populations of interconnected neurons. Such activity may not alter the abundance of a gene transcript significantly; nevertheless, the coordinated activity within transcriptional networks is being modulated dynamically to modify function.

To address this question, we applied a covariance approach using a Pearson correlation analysis, to determine how pairs of genes in mouse dorsal root ganglion (DRG) neurons are coordinately expressed in response to stimulation producing the same number of action potentials in different temporal patterns. Our analysis of 4,728 distinct gene pairs related to calcium signaling, 435,711 pairs of transcription factors, 820 pairs of voltage-gated ion channels, and 86,862 calcium-signaling genes paired with transcription factors, indicates that genes become coordinately activated by distinct action potential firing patterns. Thus, in addition to regulating the expression level of numerous genes, the temporal pattern of action potential firing profoundly modulates how genes are networked in functional pathways.

Our findings provide a deeper understanding of how nervous system development and plasticity are regulated by information coded in the temporal pattern of impulse firing in the brain. The findings are also relevant to chronic pain, as well as to the regulation of nervous system development and myelination by functional activity.
DIFFERENCES IN CHROMATIN STRUCTURE BETWEEN NEURONS AND GLIA
In collaboration with our NICHD colleague, David Clark, our research is revealing fundamental differences in chromatin structure between neurons and glia. Chromatin can be visualized by electron microscopy as regularly spaced ‘beads-on-a-string,’ in which the beads represent nucleosome cores and the string is the intervening linker DNA. Using MNase (micrococcal nuclease digestion) digestion and RNA-seq, we compared the chromatin structure of purified mouse DRG neurons, cortical oligodendrocyte precursor cells (OPCs), and cortical astrocytes. We found that DRG neurons have shorter average nucleosome spacing (approximately 165 base pairs) than either glial cells (OPCs, with approximately 182 base pair spacing) or astrocytes (with approximately 183 base pairs). The significance of such basic differences in chromatin structure between DRG neurons and these glial cells is unknown and is currently being investigated. Interestingly, the atypical nucleosome spacing of neuronal chromatin does not extend to promoter-proximal regions.

Synaptic plasticity
It is widely appreciated that there are two types of memory, short-term and long-term, and that sleep plays a critical role in memory consolidation. Gene expression is necessary to convert short-term into long-term memory, and our research concerns how signals reach the nucleus to initiate the process and which genes control strengthening and weakening of synapses in association with learning. Long-term potentiation (LTP) and long-term depression (LTD) are two widely studied forms of synaptic plasticity that can be recorded electrophysiologically in the hippocampus and are believed to represent a cellular basis for memory. We use electrophysiology, cDNA microarrays, RNA-seq, calcium imaging, and two-photon in vivo imaging to investigate the signaling pathways, genes, and proteins involved in LTP and LTD in primary cell culture and hippocampal brain slice. The work is contributing to a better understanding of how regulatory networks are controlled by appropriate patterns of impulses, leading to different forms of synaptic plasticity, and is identifying new molecular mechanisms regulating synaptic strength.

Publications

Collaborators
- Peter J. Basser, PhD, Section on Quantitative Imaging and Tissue Sciences, NICHD, Bethesda, MD
- David Clark, PhD, Section on Chromatin and Gene Expression, NICHD, Bethesda, MD
• Dumitru Iacobas, PhD, *New York Medical College, Valhalla, NY*
• Vanja Lazarevic, PhD, *Experimental Immunology Branch, Center for Cancer Research, NCI, Bethesda, MD*
• James O’Callaghan, PhD, *CDC Distinguished Consultant, NIOSH, and West Virginia University, Morgantown, WV*
• Sinisa Pajevic, PhD, *Division of Computational Bioscience, CIT, NIH, Bethesda, MD*
• Shahriar Sheikhbahaei, PhD, *Cellular and Systems Neurobiology Section, NINDS, Bethesda, MD*
• Jeffrey C. Smith, PhD, *Cellular and Systems Neurobiology Section, NINDS, Bethesda, MD*
• Kimberly Sullivan, PhD, *Boston University School of Public Health, Boston, MA*
• Hiroaki Wake, PhD, *National Institute for Basic Biology, Okazaki, Japan*

**Contact**
For more information, email fieldsd@mail.nih.gov or visit [http://nsdps.nichd.nih.gov](http://nsdps.nichd.nih.gov).
Translational Biophotonics in Developmental Disorders and Diseases

Brain imaging and spectroscopy of developmental disorders

We continued to use near-infrared spectroscopy as a measure of brain activity to study developmental trajectories of cognitive abilities in children. We examined the feasibility of functional near infrared spectroscopy (fNIRS) for the study of the mirror neuron network (MNN, also referred to as action-observation network [AON]) in a sample of 30 healthy controls who underwent a motor observation and execution paradigm while their brain activity was measured through EEG/fNIRS simultaneously. Overall, our results indicated that the parietal regions, including bilateral superior parietal lobule (SPL), bilateral inferior parietal lobule (IPL), right supra-marginal region (SMG) and right angular gyrus (AG) are candidate regions of the human AON (Figure 1). The AON is associated with the development of sophisticated social behaviors that emerge in typical human infants (e.g., complex imitation, decoding emotional states). Modeling MNN development using fNIRS (and EEG) will create a sensitive measure of deviations in social communication development before clinical behavioral deficits can be detected. To fully characterize the MNN using concurrent signals (EEG and fNIRS), we are currently conducting multimodal multiset data-fusion analysis with the goal of allowing the modalities to fully interact. After applying multiset canonical correlation analysis (mCCA) to the integrated datasets, we observed results consistent with previous literature. Preliminary results showed that action execution/observation exhibits a similar pattern of activity across regions of interest, indicating higher brain activity in regions in the left hemisphere (paracentral region, precentral region, and parietal inferior and superior regions) while subjects were performing an action. For the observation condition, higher brain activity was also found in left regions of the brain, namely the postcentral, paracentral, precentral, and parietal superior and inferior regions. This preliminary analysis is significant, because it is the first report describing the use of distinct brain metrics (hemodynamic response function and electrical activity) to characterize the MNN in the human brain. Future work includes testing the paradigm in a subset of typically developing infants and infants at risk for development/autism-spectrum disorder, who will be followed longitudinally. We will examine the developmental status of at-risk infants in relation to their initial neural data to determine whether MNN activation predicts developmental outcomes.
We finalized the project using fNIRS to examine the hemodynamic response in the prefrontal cortex (PFC) during a speech- and gesture-comprehension task [Reference 1]. Gesture abilities develop prior to speech, making gesture comprehension a detector of earlier aberrations in development. For this reason, we used both gesture and speech stimuli to examine how cortical activation may predict developmental differences. In children aged 18–36 months, we measured cortical activity by fNIRS while they were exposed to four types of stimuli: (1) meaningful gesture; (2) meaningless gesture; (3) meaningful speech; and (4) meaningless speech. Such stimuli allowed us to contrast brain activation across different types of communication and communicative intent. Children were also assessed at age three for language ability. The study showed differential activation to gesture compared with speech stimuli, as well as differential activation to meaningful versus non-meaningful stimuli. Importantly, the differences in mean activation in the left PFC in response to meaningful gesture (when controlling for meaningless gesture) at age two predicted verbal ability at age three. Differences in mean activation in response to meaningful speech compared with meaningful gesture at age two also predicted verbal ability at age three. The findings may reflect potential biomarkers for aspects of language development. Future research using larger samples of children with a wider array of language outcomes, such as children with specific language impairments, will be imperative for establishing whether this biomarker could be beneficial for early identification of aberrant language development.

We also used fNIRS to examine the underlying brain function in ornithine transcarbamylase deficiency (OTCD), the most common form of urea-cycle disorder, which is characterized by hyperammonia (HA). Using fNIRS, we examined the hemodynamics of the PFC in the OTCD population and in fraternal twins with and without OTCD. Results revealed a distinction in left PFC activation between controls and patients with OTCD, where controls showed higher task-related activation increase while performing the Stroop task. Subjects with OTCD also exhibited a bilateral increase in PFC activation. We quantified the hemodynamic variations in total hemoglobin, while twins performed the N-back working memory task [Reference 2].
Our results showed that the sibling with OTCD had higher variations in a very low frequency band (less than 0.03 Hz, related to the mechanism of cerebral autoregulation) compared with the control sibling, possibly owing to the effect of HA. Functional connectivity (FC) analysis also revealed lower inter-hemispheric FC in an OTCD sibling as the task load increased (Figure 2). Our pilot results are the first to show the utility of fNIRS in monitoring OTCD cortical hemodynamics and in indicating the possibility of inefficient neurocognitive function [Reference 3].

We are continuing to collaborate with Walter Reed Hospital on traumatic brain injury (TBI). Traumatic cerebral vascular injury (TCVI) is a frequent, but under-recognized, endophenotype of TBI. It likely contributes to functional deficits after TBI and TBI-related chronic disability, and represents an attractive target for targeted therapeutic interventions. The aim of this prospective study is to assess microvascular injury/dysfunction in chronic TBI by measuring cerebral vascular reactivity (CVR) by two methods: functional magnetic resonance imaging (fMRI) and fNIRS imaging, as each has useful features relevant to clinical utility. 42 subjects (27 chronic TBI, 15 age- and gender-matched non-TBI volunteers) were enrolled and underwent outpatient CVR testing by two methods: MRI-BOLD (BOLDscreen is an MRI-safe HD LCD monitor), and fNIRS, each with a hypercapnia challenge, a neuropsychological testing battery, and symptom-survey questionnaires. Chronic TBI subjects showed a significant reduction in global CVR compared with healthy controls. Our results show that pertinent parameters, such as mean CVR measured by fMRI and BOLD for non-TBI and TBI subjects, are different and correlate extremely well with CVR parameters obtained by fNIRS. Global CVR measured by fNIRS imaging also correlates with results obtained by MRI-BOLD. Focal CVR deficits seen on CVR maps by fMRI are also observed by fNIRS in the same areas in the frontal regions. Global CVR is significantly lower in chronic TBI patients and is reliably measured by both fMRI and fNIRS, the former with better spatial and the latter with better temporal resolution. Both methods show promise as non-invasive measures of CVR function and microvascular integrity after TBI [Reference 4].

We have begun to analyze data collected using fNIRS to validate cognitive tasks previously evaluated using fMRI. Using simultaneously collected fNIRS of the PFC and high-frequency heart-rate variability (HF-HRV), as derived from an electrocardiogram, we looked at the connection between prefrontal activation and parasympathetic nervous system activity (as measured HF-HRV) during a behavioral flexibility task (the go/no-go task).
COVID-19 point-of-care biosensor

The coronavirus disease 2019 (COVID-19) pandemic has created a challenge for researchers and healthcare professionals to design and test methods for screening and early detection of infected subjects, as well as for monitoring infected patients undergoing treatment. Using our expertise in tissue oxygenation, we are in the process of a clinical protocol approval to test a wearable multimodal biosensor. The device consists of a near-infrared spectroscopy (NIRS), a photoplethysmogram (PPG), and a thermometer sensor, capable of monitoring skin temperature, tissue oxygenation level, heart rate, and respiratory parameters. Data will be collected through a pilot study using this device in 40 healthy subjects who experienced a breathing pattern similar to that seen in pneumonia, using hypercapnia, paced breathing, and breath holding. We will identify vital parameters extracted from NIRS signals that could distinguish between normal and patterned breathing. The end product will be a point-of-care home-accessible device with Bluetooth functionality that is capable of identifying a COVID-19 infection. The study is a collaboration with Bruce Tromberg’s Section and with Babak Shadgan. We are testing the first prototype of the wearable multimodal biosensor capable of monitoring skin temperature, tissue oxygenation, respiratory, and cardiac parameters.

In the meantime, data collected by Babak Shadgan through a pilot study using this device in five healthy subjects, who experienced a breathing pattern simulating that seen in pneumonia, show encouraging results. We identified distinctive NIRS parameters that could distinguish between normal and loaded or shallow breathing. Respiratory rate (RR), respiratory rhythm (RT), breathing depth (BD), breathing interval (BI), breathing effort (BE) and inspiration slope (IS) are the respiratory-function parameters being considered in the first round of analysis; Figure 3 shows the distinctive patterns. In the next few months and as soon as the clinical protocol is approved, we will begin recruiting and will eventually apply our technology to COVID-19 patients on the NIH campus. Our ultimate goal is to use artificial intelligence and machine learning to identify a pattern of NIRS respiration and tissue oxygenation that would be specific to COVID-19.

Placenta oxygenation: from basics to point of care

Placental oxygenation plays a crucial role in a healthy pregnancy and its outcome. Defects in the placenta that affect placental oxygenation can cause preeclampsia, intrauterine growth restriction, fetal hypoxia, asphyxia, and cerebral palsy. A fast and non-invasive method that measures placental oxygenation quantitatively is necessary to detect such abnormalities. Current methods are either time consuming or not patient-friendly.
We therefore developed a wearable device using NIRS that monitors anterior placenta oxygenation non-invasively and dynamically. The device uses two light sources, with 760 nm and 840 nm wavelengths, because they are sensitive to changes in blood oxyhemoglobin and deoxyhemoglobin. It consists of two photodiodes as detectors and six LED light sources, which are placed at six different distances, from 10 to 60 mm, from the LEDs. The different source and detector distances help us scan different tissue depths in order to distinguish between placental oxygenation and oxygenation of maternal layers. Also, the probe has a flexible geometry that enables us to place it in proper contact with the skin.

For the \textit{in vivo} study, we tested the device on subjects in Detroit in collaboration with the Maternal-Fetal Medicine, Imaging, and Behavioral Development Affinity Group (Roberto Romero) at NICHD, Wayne State University, and with USUHS. The study focuses on baseline placental oxygenation for normal term pregnancies scheduled for cesarean section; ultra-sound imaging gives us the fat and uterus thicknesses that we need for the analysis. So far, we have measured placental oxygenation in 12 healthy, singleton, pregnant volunteers (33.3±3.6 weeks pregnant). We are in the process of completing our measurements on a total of 40 subjects to obtain adequate statistical power. The placental oxygenation calculated from two source-detector separations (30mm and 40mm) for this group of 12 subjects ranges from 68% to 89%. However, we found that the calculated placental oxygenation is positively correlated with the thickness of the fat layer: pregnant women with a thicker fat layer display higher placental oxygenation. We believe that the correlation was caused by the highly scattering characteristic of the fat.

We are thus now performing a Monte Carlo simulation on a five-layer model to correct for the effect of maternal layers such as fat on placental oxygenation. The simulations are based on thickness and on both the scattering and absorption coefficients of all maternal layers (dermis, epidermis, fast, uterus) and placenta. Although the placenta is an essential organ for fetal development and successful reproduction, of these it is the least-studied organ. We therefore also measured the scattering coefficients of the human placenta in the range of 659 to 840nm, using a well-established frequency domain diffuse optical spectroscopic system (DOSI) and a lab-designed diffuse reflectance device (DRS). Measurements were performed on eight placentas obtained after cesarean deliveries. We then calculated absorption and scattering coefficients calculated from the measured reflectance using the random-walk theory for DRS and a frequency-domain algorithm for DOSI. Average reduced scattering coefficient ($\mu_s'$) was 0.943 ± 0.015 mm$^{-1}$ at 760 nm and 0.831 ± 0.009 mm$^{-1}$ at 840 nm. A power law with an exponent of 1.426 describes accurately changes of human placental scattering coefficient as a function of wavelength. Such scattering coefficients can be used to improve measurements of placental oxygen saturation [Reference 5].

Along with the \textit{in vivo} studies, we are studying placental oxygenation at the cellular level using a novel biophotonics method named dynamic full-field optical coherence tomography (DFFOCT) and in HeLa cells with manually changed oxygenation. The preliminary results established the ability of DFFOCT to detect the changes in intracellular activity for different oxygen levels. HeLa cell samples were treated with Triton X-100, which causes membrane permeabilization, and paraformaldehyde, which causes cell fixation. We imaged untreated and treated samples using DFFOCT to determine whether it could detect cellular activity. We were able to isolate cellular signals from the environment and measure changes in cellular activity following various inhibition treatments, highlighting the potential of DFFOCT to uncover new information about dynamic intracellular fluctuations during various cellular processes. Future experiments with targeted cellular treatments can be conducted to further characterize cellular activity. To identify the biological causes of the
signal from untreated samples, we plan controlled experiments involving the suppression of cellular energetics by mitochondrial inhibitors and glucose decouplers. Cellular energetics are essential for large polymer buildup, disassembly, movement within the cell, and small protein activity.

**Tissue characterization and function**

We are investigating photonic techniques to elucidate biomarkers for the diagnosis of diseases or the assessment of treatment outcome across a variety of conditions. We are assessing facial plethora in Cushing’s syndrome (CS), as it was one of the earliest described clinical features of the disease. In collaboration with Constantine Stratakis, we quantified changes in facial plethora in CS as an early assessment of cure. We performed noninvasive multispectral NIR imaging on the right cheek of patients before and after surgery. Patients were defined as cured by postoperative levels of plasma cortisol of less than 3 mcg/ml and/or by adrenocortical insufficiency, for which they received replacement therapy. Results indicate that a reduction in facial plethora after surgery, as evidenced by a reduction in blood volume fraction, is correlated with the cure of CS. The first set of results were published in 2015. In our follow-up paper [Reference 6], we also showed that the water-content fraction can be used as a new biomarker of early cure in patients with CS. We recorded data for 29 new patients, and follow-up imaging was conducted for 26 patients. We also developed and tested a new hand-held system that has improved performance over the existing portable system. We plan to use the system as a point-of-care imaging device. In brief, the new imager uses a high-resolution complementary metal oxide semiconductor (CMOS) camera with on-chip filters. Images are acquired simultaneously at eight different near-infrared wavelengths (700–980 nm). Our graphical user interface (Figure 3c) now supports both portable and the hand-held multispectral imagers.

Annually, about 15 million preterm infants are born in the world. Of these, about 1 million die before the age of five because of complications resulting from their premature birth. Given that the high incidence of preterm birth (PTB) is partially the result of the lack of effective diagnostic modalities, methodologies are needed to determine the risk of PTB. We proposed a non-invasive tool based on polarized-light imaging aimed at measuring the organization of collagen in the cervix. Cervical collagen has been shown to remodel with the approach of parturition. We used a full-field Mueller matrix polarimetric colposcope to assess and compare cervical collagen content and structure in nonpregnant and pregnant women. The local collagen directional azimuth was used, and we imaged a total of eight cervices. In a continued collaboration with Jessica Ramella-Roman on preterm pregnancy complications, we used the Preterm Imaging system based on colposcopy to characterize uterine cervix structure in a longitudinal study of low-risk and high-risk (i.e., prior PTB or a sonographic short cervix) patients. Polarization imaging is an effective tool to measure optical anisotropy in birefringent materials, such as the cervix’s extracellular matrix, and to predict cervical ripening. For this reason, it has potential to predict preterm birth. Through our collaborations with Roberto Romero’s Branch and with Jessica Ramella-Roman, we will test the system in a control population and those with PTB prevalence [Reference 5].

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Publications


Collaborators

- Franck Amyot, PhD, *Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD*
- Mehran Armand, PhD, *The Johns Hopkins University, Baltimore, MD*
- Claude Boccara, PhD, *École Supérieure de Physique et de Chimie Industrielles, Paris, France*
- Andrea Gropman, MD, *Children’s National Health System, Washington, DC*
- Sonia S. Hassan, MD, *Wayne State University School of Medicine, Detroit, MI*
- Tom Pohida, MS, *Division of Computational Bioscience, CIT, NIH, Bethesda, MD*
- Jay Knutson, PhD, *Laboratory of Molecular Biophysics, NHLBI, Bethesda, MD*
- Randall Pursley, *Signal Processing and Instrumentation Section, CIT, NIH, Bethesda, MD*
- Jessica C. Ramella-Roman, PhD, *Florida International University, Miami, FL*
- Roberto Romero-Galue, MD, *Perinatology Research Branch, NICHD, Detroit, MI*
- Dan Sackett, PhD, *Division of Basic and Translational Biophysics, NICHD, Bethesda, MD*
- Babak Shadgan, MD, MSc, PhD, *University of British Columbia, Vancouver, Canada*
- Constantine Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*
- Audrey Thurm, PhD, *Pediatrics & Developmental Neuropsychiatry Branch, NIMH, Bethesda, MD*
- Bruce Tromberg, PhD, *Section on Biomedical Optics, NICHD, Bethesda, MD*
- Eric Wassermann, MD, *Cognitive Neuroscience Section, NINDS, Bethesda, MD*

Contact

For more information, email gandjbaa@mail.nih.gov or visit https://irp.nih.gov/pi/amir-gandjbakhche.
Transcriptional and Translational Regulatory Mechanisms in Nutrient Control of Gene Expression

We study the fundamental mechanisms involved in the assembly and function of translation initiation complexes for protein synthesis, using yeast as a model system in order to exploit its powerful combination of genetics and biochemistry. The translation initiation pathway produces an 80S ribosome bound to mRNA, with methionyl initiator tRNA (tRNAi) base-paired to the AUG start codon. The tRNAi is recruited to the small (40S) subunit in a ternary complex (TC) with the GTP-bound eukaryotic initiation factor eIF2 to produce the 43S preinitiation complex (PIC) in a reaction stimulated by eIFs 1, 1A, 3, and 5. The 43S PIC attaches to the 5' end of mRNA, facilitated by the cap-binding complex eIF4F (comprising eIF4E, eIF4G, and the RNA helicase eIF4A) and poly(A)-binding protein (PABP) bound to the poly(A) tail, and scans the 5' untranslated region (UTR) for the AUG start codon. Scanning is promoted by eIF1 and eIF1A, which induce an open conformation of the 40S and rapid TC binding in a conformation suitable for the scanning of successive triplets entering the ribosomal P site (P-out), and by eIF4F and other RNA helicases, such as Ded1 and its paralog Dbp1, that remove secondary structure in the 5'UTR. AUG recognition evokes tighter binding of the TC in the P-in state and irreversible GTP hydrolysis by eIF2, dependent on the GTPase-activating protein (GAP) eIF5, releasing eIF2-GDP from the PIC, with tRNAi remaining in the P site. Joining of the 60S subunit produces the 80S initiation complex ready for protein synthesis. Our current aims in this research area are to: (1) elucidate the functions of eIF1, eIF5, eIF3, and 40S ribosomal proteins in TC recruitment and start-codon recognition; (2) identify distinct functions of the RNA helicases eIF4A (and its cofactors eIF4G/eIF4B), Ded1, and Dbp1, and of the poly(A)-binding protein (PABP) in mRNA activation, 48S PIC assembly, and scanning in vivo; (3) uncover the mechanisms of translational repression and regulation of mRNA abundance by the repressors Scd6, Pat1, the helicase Dhh1, and the mRNA-decapping enzyme Dcp2; (4) elucidate the regulation of Ded1, eIF4G, and Dhh1 functions in response to nutrient limitation or stress; (5) elucidate the in vivo functions of yeast eIF2D orthologs and of the MCT-1/DENR complex in 40S ribosome recycling at stop codons and reinitiation in 3' untranslated regions in vivo; and (6) elucidate the roles of yeast orthologs of eIF2A and eIF2D in eIF2-independent initiation of translation in stress conditions.

We also analyze the regulation of amino acid-biosynthetic genes in budding yeast as a means of dissecting fundamental mechanisms...
of transcriptional control of gene expression. During amino acid limitation, transcription of such genes is coordinately induced by the activator Gcn4 as the result of induction of Gcn4 at the translational level. The eviction of nucleosomes that occlude promoter DNA sequences and block access by RNA polymerase is thought to be a rate-limiting step for transcriptional activation. Previous studies implicated certain histone chaperones, ATP–dependent chromatin-remodeling complexes, or histone acetyltransferase (HAT) complexes in eviction of promoter nucleosomes at certain yeast genes, but it is unclear whether these co-factors function at Gcn4 target genes. Our aim is to elucidate the full set of co-factors that participate in promoter nucleosome eviction at Gcn4 target genes, their involvement in this process genome-wide, and the transcriptional consequences of defective nucleosome eviction. Functional cooperation among the chromatin-remodeling complexes SWI/SNF, RSC, and Ino80, as well as the HAT complexes SAGA, NuA4, NuA3, and Rtt109/Asf1, in these processes are under study. We recently discovered that Gcn4 can activate transcription from binding sites within the coding sequences (CDS) of its target genes, inducing internal subgenic sense and antisense (AS) transcripts in addition to the conventional full-length transcripts that initiate 5′ of the CDS; and we are probing both the mechanism and possible regulatory functions of these internal AS transcripts, as well as the roles of co-transcriptional histone methylation, nucleosome reassembly, and mRNA decay enzymes in controlling their synthesis and abundance. We are also probing mechanisms involved in the asymmetric transcriptional induction of genes belonging to pairs of divergently oriented genes where only one gene responds to Gcn4 binding at the shared upstream activation sequences (enhancer).

**eIF2α interactions with mRNA control accurate start-codon selection by the translation preinitiation complex.**

Comparison of previous cryo-EM structures of 48S PICs in open, scanning-conducive or closed, arrested conformations revealed interactions unique to the closed complex between Arg residues R55 and R57 of domain 1 of the α-subunit of eIF2 (eIF2α-D1) with mRNA nucleotides 5′ of the AUG codon, including the –3 residue of the “Kozak” context (the Kozak consensus sequence is a nucleic acid motif that functions as the protein translation initiation site in most eukaryotic mRNA transcripts). We showed that substitutions of R55 and R57 reduce recognition of the poor-context AUG codon for SUI1 mRNA (encoding eIF1) and also UUG start codons in Sui– cells (the Ssu– phenotype). We further showed that the R55G-R57E Ssu– substitutions destabilize TC binding to 48S PICs reconstituted with mRNA with a UUG start codon in the *in vitro* reconstituted system, in the manner expected from specific destabilization of the closed complex at a near-cognate codon. Interestingly, residue R53 of eIF2α-D1 interacts with rRNA residues exclusively in the open complex; we found that the R53E substitution enhances initiation at UUG codons (the Sui– phenotype) and the poor-context SUI1 AUG, and also confers the Gcd– phenotype, indicating constitutively depressed translation of *GCN4* mRNA, which results from slow recruitment of the TC to scanning 40S subunits engaged in re-initiation on this mRNA *in vivo*. In the reconstituted system, R53E stabilized TC binding to UUG complexes while simultaneously reducing the on-rate of TC loading, all in the manner predicted for specific destabilization of the open complex and shift towards the closed state. We conclude that distinct interactions of eIF2α-D1 with the rRNA or mRNA stabilize first the open, and then the closed, conformation of the PIC to regulate the accuracy and efficiency of start codon selection *in vivo* [Reference 5].

**eIF1 discriminates against suboptimal initiation sites to prevent excessive uORF translation genome-wide.**

To uncover the genome-wide role of eIF1 in promoting accurate start-codon selection, we conducted ribosome profiling (deep-sequencing of all native ribosome-protected mRNA fragments) of the eIF1–L96P mutant,
which is impaired for interactions with eIF3c, eIF5, and the 40S subunit, expected to occur in the scanning PIC. The profiling data indicated that L96P increases initiation at near-cognate start codons (NCCs) that initiate N-terminal extensions for several proteins, which we confirmed by reporter assays. L96P also increased translation of hundreds of upstream open reading frames (uORFs) initiated by NCCs. We also observed increased utilization of poor-context AUGs, both at AUG-initiated uORFs (that exhibit poor context as a group) and at the small fraction of main CDSs with poor-context AUGs. Interestingly, L96P leads to reduced translation of a subset of mRNAs in a manner associated with increased uORF translation, including carboxypeptidase A1 (CPA1) mRNA, which is negatively regulated by its single AUG uORF. Thus, eIF1 acts broadly to discriminate against NCC start codons and poor-context AUGs; impairing this function can increase the repressive effects of uORFs and alter the ratios of protein isoforms translated from the same mRNAs by NCC versus AUG start codons [Reference 1].

Functional interplay between RNA helicases Ded1 and Dbp1 in stimulating translation of structured mRNAs in vivo

RNA helicases eIF4A and Ded1 are believed to resolve mRNA structures that impede ribosome attachment or scanning to the start codon. By ribosome profiling of mutations in Ded1 or eIF4A, we previously found that inactivation of Ded1 reduced the relative translational efficiencies (TEs) of over 600 mRNAs characterized by relatively long and structured 5′UTRs, whereas inactivation of eIF4A similarly affected only about 40 mRNAs. Thus, Ded1 is critically required for PIC attachment and scanning through secondary structures, while eIF4A promotes a step of initiation common to all mRNAs. We reconstituted the function of Ded1 in a purified system by showing that Ded1 accelerates 48S PIC assembly to a greater extent for Ded1–hyperdependent versus Ded1–hypodependent mRNAs identified by ribosome profiling, and that eliminating 5′UTR stem-loop structures enhanced Ded1–independent recruitment and diminished Ded1 acceleration of 48S assembly. To illuminate the in vivo function of the Ded1 paralog Dbp1, we conducted ribosome profiling of dbp1Δ and dbp1Δded1 double mutants. The results indicate that Dbp1 functionally cooperates with Ded1 throughout the translome in stimulating translation of mRNAs with long, structure-prone 5′UTRs, as the TE reductions in the double mutant generally exceed those in the ded1 single mutant. For many such mRNAs, Dbp1 largely masks the involvement of Ded1. Importantly, Dbp1 mimics Ded1 in accelerating 48S preinitiation complex (PIC) assembly in the reconstituted system on Ded1–hyperdependent mRNAs with structured 5′UTRs. Using the recently developed method of TCP-seq for genome-wide profiling of 40S subunits, we quantified PIC occupancies in 5′UTRs and found that 40S subunits tend to accumulate in the 5′UTRs of mRNAs in the helicase mutants, particularly for mRNAs judged to be Ded1/dbp1–hyperdependent by 80S ribosome profiling, thus providing direct evidence that Ded1/Dbp1 stimulate scanning through structured 5′UTRs in vivo to enhance translation. We also uncovered cooperation between these helicases in promoting 43S PIC attachment to a subset of helicase-dependent mRNAs, which exhibit reduced 40S occupancies in 5′UTRs in the helicase double mutant [Reference 6].

eIF4A and eIF4E interactions with distinct residues of the Ded1 N-terminus stimulate Ded1 function in translation initiation in vivo.

Binding of eIF4F to the mRNA cap structure enhances recruitment of the 43S PIC to the 5′end and subsequent scanning of the 5′UTR. Ded1 physically interacts with eIF4A and the eIF4G subunit of eIF4F, and eIF4A and eIF4G can both stimulate unwinding of a model RNA substrate by Ded1 in vitro. Previously, we showed that the Ded1 C-terminal domain (CTD) and its two interacting domains in eIF4G, dubbed RNA2 and RNA3, and the Ded1 N-terminal domain (NTD) that interacts with eIF4A, all enhance Ded1 stimulation of 48S PIC assembly
in the reconstituted *in vitro* system. Ded1 also interacts with eIF4E; however the binding sites for eIF4A and eIF4E in the Ded1–NTD were unknown. By substituting blocks of conserved residues in the Ded1–NTD, we found that alanine replacements of residues 21–27 and 51–57 reduce Ded1 binding to eIF4A *in vitro*, impair association between native Ded1 and eIF4A in cell extracts, reduce cell growth, bulk translation initiation, and translation of Ded1–hyperdependent reporter mRNAs harboring stem-loop insertions. Overexpressing eIF4A diminished the growth defects for each single substitution, but not for the 21–27/51–57 double substitution, which is null for eIF4A binding, supporting the importance of Ded1–NTD/eIF4A interaction in cells. Substituting the non-overlapping residues 59–65 and 83–89 reduced Ded1–NTD binding to eIF4E *in vitro*, as well as Ded1–eIF4E association in extracts, and conferred reduced translation of the Ded1–hyperdependent reporters. Combining all four NTD substitutions conferred an additive growth defect indistinguishable from deletion of the NTD, suggesting that eIF4A/eIF4E binding is the key *in vivo* function of the Ded1 NTD. Deleting the Ded1–CTD impairs growth only when combined with NTD substitutions, implying that the Ded1–CTD interaction with eIF4G is dispensable when Ded1 can interact with eIF4A and eIF4E. In the reconstituted system, the Ded1 NTD substitutions that eliminate eIF4A binding reduce the maximal rate of 48S PIC assembly on a Ded1–dependent mRNA harboring a 5′UTR SL, and also increase the amount of Ded1 required to achieve the half-maximal rate (K1/2). Disruption of the Ded1–NTD/eIF4E interaction has a similar effect of elevating the Ded1 K1/2 for 48S assembly. The findings support the notion that Ded1 NTD interactions with eIF4A and eIF4E stabilize a Ded1–eIF4E–eIF4G–eIF4A quaternary complex that enhances Ded1's ability to resolve secondary structures in 5′UTRs [Reference 2].

**Chromatin remodeler (CR) Ino80C acts independently of histone variant H2A.Z to evict promoter nucleosomes and stimulate transcription of highly expressed genes.**

The CR Ino80C was found to be sufficient for reconstituting a near-native nucleosomal organization *in vitro*, and was also implicated in nucleosome editing to replace the histone variant H2A.Z (encoded by *HTZ1*) with canonical H2A. The removal of an H2A.Z:H2B dimer by Ino80C could render the partially disassembled nucleosome more susceptible to eviction; however, a prominent role for Ino80C in promoter nucleosome eviction had not been reported. By ChIP-seq analysis of the histone H3 and RNA polymerase II (Pol II) in an *ino80Δ* mutant lacking the Ino80C catalytic subunit, we found that Ino80C functions on par with SWI/SNF in eviction of promoter nucleosomes and transcriptional activation of Gcn4 target genes, and plays a much greater role than the chromatin-remodeling complexes SWI/SNF at a group of several hundred Ino80C–hyperdependent genes. Compared with both SWI/SNF and RSC, Ino80C generally functions over a wider interval spanning the -1 and +1 nucleosomes, the nucleosome-depleted-region (NDR), and NDR–proximal genic nucleosomes. At Gcn4 target genes, the degree of nucleosome eviction defect is correlated with the reduction in transcription; and ChIP-seq analysis of the GTF TATA–binding protein (TBP) revealed that defects in nucleosome eviction are accompanied by reduced promoter occupancies of TBP, and hence PIC assembly. ChIP-seq analysis of Ino80C itself shows that Ino80C is enriched at both Gcn4 target genes and Ino80C–hyperdependent genes. Thus, Ino80C cooperates with RSC and SWI/SNF in evicting promoter nucleosomes to enhance PIC assembly and transcription at many highly expressed genes [Reference 4] (see image below).

If Ino80C enhances nucleosome eviction strictly in the course of editing H2A.Z-H2B dimers, then deleting *HTZ1* should mimic the effect of deleting *Ino80* on promoter nucleosome eviction. Moreover, depleting Ino80 should have no effect on nucleosome occupancies in cells lacking HTZ1. At odds with these predictions, we found that the *htz1Δ* mutation has much smaller effects than *ino80Δ* on eviction of promoter nucleosomes.

**SECTION ON NUTRIENT CONTROL OF GENE EXPRESSION**
Moreover, depleting Ino80 from the nucleus by “anchor-away” impaired histone eviction in cells lacking HTZ1. Thus, Ino80C can function like the SWI/SNF family members SWI/SNF and RSC in promoting chromatin access independently of nucleosome editing [Reference 4].

Function and regulation of Gcn4 binding within coding regions in activating internal and canonical 5 promoters in yeast

We are also interested in determining the role of promoter nucleosome eviction in controlling binding of Gcn4 itself upstream from the promoters of its target genes. We thus set out to define all binding sites for Gcn4 throughout the genome in wild-type (WT) cells. ChIP-seq analysis of Gcn4 binding revealed 546 genomic sites occupied by Gcn4 in starved cells, representing only 30% of all genomic sequences with significant matches to the consensus Gcn4–binding motif. Analysis of nucleosome occupancy data from MNase-seq analysis revealed that the distance of a motif from the nearest nucleosome dyad and its match to the consensus sequence are the major determinants of Gcn4 binding in vivo. Surprisingly, only 40% of the bound sites are in promoters, and analysis of genome-wide mRNA expression data and ChIP-seq analysis of Pol II in starvation conditions indicates that only 60% of such promoter-located Gcn4 occupancy peaks activate transcription, indicating extensive negative control over Gcn4 function. Remarkably, most of the remaining 300 Gcn4–bound motifs reside within coding sequences (CDS), with 75 representing the only bound motif in the vicinity of a known Gcn4–induced gene. RNA-seq analysis revealed that many such unconventional Gcn4 occupancy peaks map between divergent antisense (AS) and sub-genic sense transcripts induced from within CDS under starvation conditions, and are also located adjacent to starvation-induced TBP occupancy peaks detected by ChIP-seq analysis, findings that are consistent with Gcn4 activation of cryptic, bidirectional internal promoters at these genes. Mutational analysis confirmed that Gcn4–bound motifs within CDS can activate both sub-genic and

FIGURE 1. Model for cooperation between the chromatin remodelers Ino80C, SWI/SNF, and RSC in promoter nucleosome disassembly at Gcn4–induced genes

In non-inducing conditions, Ino80C functions in evicting nucleosomes from the promoter region. Induction of Gcn4 leads to additional recruitment of Ino80C, as well as SWI/SNF and RSC, to augment eviction of promoter nucleosomes and thereby increase access of general transcription factors, including TBP, to stimulate PIC assembly by Pol II. Nucleosomes are depicted as multicolored cylinders; cylinders with faint shades indicate nucleosomes undergoing eviction.
full-length transcripts from the same or adjacent genes, demonstrating that functional Gcn4 binding is not confined to promoters. Our results show that internal promoters can be regulated by a well-defined activator that also functions at conventional 5′-positioned promoters.

Current experiments are aimed at determining whether induction of internal AS promoters by Gcn4 serves to dampen its activation of the upstream promoters for the full-length sense transcripts at these genes, via collisions between Pol II molecules transcribing from opposite DNA strands, or by co-transcriptional methylation by the histone-lysine N-methyltransferases Set1 or Set2, with attendant deacetylation of the upstream promoter nucleosomes. We are also examining the roles of Set1 and Set2, and the histone chaperone Spt6 in suppressing cryptic internal promoters activated by Gcn4, and of the nuclear exosome and the nonsense-mediated decay (NMD) pathway in degradation of the AS transcripts. We are also probing the roles of chromatin remodelers, HAT complexes, and general regulatory factors in promoting Gcn4 binding to its target sequences in vivo.

**Publications**


**Collaborators**

- Stuart Archer, PhD, *Monash Bioinformatics Platform, Monash University, Australia.*
- David Clark, PhD, *Section on Chromatin and Gene Expression, NICHD, Bethesda, MD*
- Chhabi Govind, PhD, *Oakland University, Rochester, MI*
- Jon Lorsch, PhD, *Laboratory on the Mechanism and Regulation of Protein Synthesis, NICHD, Bethesda, MD*
- Thomas Preiss, PhD, *The John Curtin School of Medical Research, The Australian National University, Canberra, Australia*
- Venkatraman Ramakrishnan, PhD, *MRC Laboratory of Molecular Biology, Cambridge, United Kingdom*

**Contact**

For more information, email ahinnebusch@nih.gov or visit [https://www.nichd.nih.gov/research/atNICHD/Investigators/hinnebusch](https://www.nichd.nih.gov/research/atNICHD/Investigators/hinnebusch).
Molecular Nature and Functional Role of Dendritic Voltage-Gated Ion Channels

The central nervous system (CNS) underlies all our experiences, actions, emotions, knowledge, and memories. With billions of neurons each firing hundreds of times per second, the complexity of the brain is stunning. To pare down the task of understanding something so complex, our research approach calls for studying the workings of a single central neuron: the pyramidal neuron from the CA1 region of the hippocampus. The hippocampus is essential for long-term memory in humans and is among the first brain regions affected by epilepsy and Alzheimer’s disease. To understand how the hippocampus stores and processes information, we focus on one of its principal cell types, the CA1 pyramidal neuron. Each pyramidal neuron in the CA1 region of the hippocampus receives tens of thousands of inputs onto its dendrites, and it is commonly thought that information is stored by altering the strength of individual synapses (synaptic plasticity). Recent evidence suggests that the regulation of synaptic surface expression of glutamate receptors can, in part, determine synaptic strength. However, the dendrites contain an abundance of ion channels that are involved in receiving, transforming, and relaying information in the dendrites, adding an additional layer of complexity to neuronal information processing.

We found that the A-type potassium channel subunit Kv4.2 is highly expressed in the dendritic regions of CA1 neurons in the hippocampus and, as one of the primary regulators of dendritic excitability, plays a pivotal role in information processing. Kv4.2 is targeted for modulation during the types of plasticity thought to underlie learning and memory. Moreover, we found that the functional expression level of Kv4.2 regulates the subtype expression of NMDA-type glutamate receptors, the predominant molecular devices controlling synaptic plasticity and memory. We are currently following up on these findings with more detailed investigations into the mechanisms of activity-dependent Kv4.2 regulation. In addition, we have begun to investigate the role of dendritic voltage-gated potassium and calcium channels in neuronal development and developmental disorders.

Role of voltage-gated ion channels in synaptic development and disease

REGULATION OF POTASSIUM CHANNEL TRAFFICKING AND FUNCTION

Kv4.2 channels, the major contributors to somatodendritic A-type
potassium channels, are key determinants of dendritic excitability and integration, spike timing-dependent plasticity, and long-term potentiation. Downregulation of Kv4.2 channel expression occurs following hippocampal seizures and in epilepsy, suggesting that A-type currents may be targets for novel therapeutics. To identify Kv4.2–binding proteins, Jiahua Hu employed a tandem affinity purification (TAP) approach to isolate the Kv4.2 protein complex from hippocampal neurons. Mass-spectrometry (MS) analysis identified known proteins such as KChIP (see below) family members and DPP6/10. The TAP–MS assay also identified an isomerase as a binding partner of Kv4.2. The binding was confirmed by brain co-immunoprecipitation, co-expression in HEK293T cells, and peptide pull-down in vitro. The isomerase binds to a specific Kv4.2 site, and the association is regulated by neuronal activity and seizure.

We recently identified a novel molecular cascade initiated by the activation of p38 kinase and subsequent isomerization of a C-terminal motif (T607) in Kv4.2, which triggers dissociation from its auxiliary subunit DPP6, a reduction I_A (type A potassium current), and an increase in neuronal excitability. The phosphorylation of the Kv4.2 T607 site is induced by novel environment exposure or seizure and is mediated by p38 MAPK (mitogen-activated protein kinase) but not by ERK MAPK. To investigate the consequences of this cascade on behavior and neuronal physiology, we used CRISP-Cas9 techniques to generate a knockin mouse in which the isomerase binding site is specifically abolished (Kv4.2TA). The mice are viable and appear normal, although activity-dependent dissociation of the Kv4.2-DPP6 complex is impaired.

Cole Malloy used patch-clamp electrophysiology in pyramidal cells of hippocampal slices from Kv4.2TA and wild-type (WT) mice to decipher the role of p38-Pin1 (a peptidyl-prolyl isomerase)–mediated regulation of Kv4.2 on neuronal excitability. He found that Kv4.2TA cells displayed a reduction in action potential (AP) firing compared with WT in response to somatic current injections. The reduced excitability can be traced to increased Kv4.2–mediated current in Kv4.2TA cells in outside-out somatic patches. Pharmacological block of both p38 kinase and Pin1 in WT recapitulated the impact of the mutation on neuronal firing properties and I_A, confirming the specificity of this cascade as underlying these effects.

To detect how such alterations in neuronal physiology are manifested in behavioral changes, Jiahua Hu performed a battery of tests to probe seizure susceptibility and learning and memory capability. In response to intraperitoneal (IP) kainic acid injection, Kv4.2TA mice exhibited reduced seizure intensity over an hour-long period compared with WT mice. The reduced seizure intensity could also be recapitulated in WT with pharmacological block of p38 kinase. We thus identified a novel signaling cascade, which can be a target for therapeutic intervention to mitigate seizure intensity in epilepsy by reducing Kv4.2 downregulation.

Furthermore, Kv4.2TA mice exhibit normal initial learning and memory in the Morris Water maze. However, they exhibited better ‘reversal’ learning in the Morris Water maze (a measure of cognitive flexibility) than did WT mice. In the operant reversal lever press, the Kv4.2TA mice displayed improved reversal learning. The data strongly support the idea that activity-dependent regulation of Kv4.2 plays an important role in cognitive flexibility, the ability to appropriately adjust one’s behavior to a changing environment, which is impaired in various neuro-developmental disorders, such as the autism spectrum disorder. In light of the findings that Kv4.2TA mice exhibit enhanced cognitive flexibility, our ongoing experiments utilize whole-cell recordings from pyramidal neurons in hippocampal slice to investigate potential differences in the synaptic properties of WT and Kv4.2TA mice. Collectively, the experiments will reveal the cellular mechanisms underlying the reversal learning phenotype in Kv4.2TA mice and will provide further insight into mechanisms impacting cognitive flexibility.
CA\textsuperscript{2+} REGULATION OF POTASSIUM CHANNEL FUNCTION

Jonathan Murphy found that Ca\textsuperscript{2+} entry mediated by the voltage-gated Ca\textsuperscript{2+} channel subunit Cav2.3 regulates Kv4.2 function both in a heterologous expression system and, endogenously, in CA1 pyramidal neurons through Ca\textsuperscript{2+} binding to auxiliary subunits known as K\textsuperscript{+} channel-interacting proteins (KChIPs). KChIPs are calcium-sensing molecules that containing four EF-hands (motif with helix-loop-helix topology), and which are dysregulated in several diseases and disorders, including epilepsy, Huntington’s disease, and Alzheimer’s disease. He characterized a KChIP–independent interaction between Cav2.3 and Kv4.2 using immunofluorescence co-localization, co-immunoprecipitation, electron microscopy, FRAP (fluorescence recovery after photobleaching), and FRET (fluorescence resonance energy transfer). We found that Ca\textsuperscript{2+} entry via Cav2.3 increases Kv4.2–mediated whole-cell current, partly as a result of an increase in Kv4.2 surface expression. In hippocampal neurons, pharmacological block of Cav2.3 reduced whole-cell I\textsubscript{A} by about 33%. We also found an approximately 20% reduction in whole-cell I\textsubscript{A} in Cav2.3 knockout (KO) mouse neurons with a loss of the characteristic dendritic I\textsubscript{A} gradient. Furthermore, we found that the Cav2.3–Kv4.2 complex regulates the size of synaptic currents and spine Ca\textsuperscript{2+} transients. The results reveal an intermolecular Cav2.3–Kv4.2 complex that impacts synaptic integration in CA1 hippocampal neurons.

The KChIP protein, but not mRNA expression, has been shown to be reduced in Kv4.2 KO mouse brains, suggesting increased KChIP protein degradation in the absence of Kv4.2. We hypothesized that KChIP protein degradation is dependent on binding to Kv4.2 and that KChIP protein degradation increases in the absence of Kv4.2. We aimed to elucidate the molecular mechanism of KChIP protein degradation and its effect on Kv4.2 protein levels and function. Joe Krzeski identified the pathway through which KChIP is degraded and a novel function for KChIP regulation of Kv4.2 in HEK293 cells. A mechanistic understanding of KChIP protein degradation is important, as it may lead to new therapeutic strategies to treat diseases in which KChIPs are dysregulated.

DPP6 PLAYS A ROLE IN BRAIN DEVELOPMENT, FUNCTION, AND BEHAVIOR.

We previously showed that the Kv4 auxiliary subunit DPP6 has a novel function in regulating dendritic filopodia formation and stability, affecting synaptic development and function. In 2018, we reported that DPP6–KO mice are impaired in hippocampus-dependent learning and memory, with smaller brain size and weight. Recently, using immunofluorescence and electron microscopy, in a project led by Lin Lin, we discovered a novel structure in hippocampal area CA1 that was significantly more prevalent in DPP6–KO mice than in WT mice of the same age and that the structures were observed earlier in development in DPP6–KO mice, appearing as clusters of large puncta that colocalized the neuronal proteins NeuN, synaptophysin, and chromogranin A. Electron microscopy revealed that the structures are abnormal, enlarged presynaptic swellings filled with mainly fibrous material, with occasional peripheral, presynaptic active zones forming synapses. We found diagnostic biomarkers of Alzheimer’s disease present in abnormal levels in DPP6–KO mice, including accumulation of amyloid β and amyloid precursor protein (APP) in the hippocampal CA1 area and a significant increase in expression of hyper-phosphorylated tau. The amyloid β and phosphorylated tau pathologies were associated with neuro-inflammation characterized by activation of microglia and astrocytes. Multiplex cytokine array detection with WT and DPP6–KO mouse blood serum showed that levels of pro-inflammatory or anti-inflammatory cytokines increased in aged DPP6–KO mice. We also found that the presence of activated astrocytes and microglia was significantly increased in DPP6–KO brain sections and that DPP6-KO mice displayed circadian dysfunction, a common symptom of Alzheimer’s disease. Together, the results indicate that DPP6–KO mice show symptoms of enhanced neurodegeneration reminiscent of Alzheimer’s disease and that they are associated with a novel structure resulting from synapse loss and neuronal death.
Publications

Collaborators
- Heather Cameron, Section on Neuroplasticity, NIMH, Bethesda, MD
- Avindra Nath, MD, Translational Neuroscience Center, NINDS, Bethesda, MD
- Constantine A. Stratakis, MD, D(med)Sci, Section on Endocrinology and Genetics, NICHD, Bethesda, MD

Contact
For more information, email hoffmand@mail.nih.gov or visit http://hoffmanlab.nichd.nih.gov.
Control of Gene Expression during Development

During development and differentiation, genes become competent to be expressed or are stably silenced in an epigenetically heritable manner. The selective activation/repression of genes leads to differentiation of tissue types. Much evidence supports the model in which modifications of histones in chromatin contribute substantially to determining whether a gene is expressed. Two groups of genes, the Polycomb group (PcG) and Trithorax group (TrxG), are important for inheritance of the silenced and active chromatin state, respectively. In Drosophila, regulatory elements called Polycomb group response elements (PREs) are required for the recruitment of chromatin-modifying PcG protein complexes. TrxG proteins may act through the same or overlapping cis-acting sequences. Our group aims to understand how PcG and TrxG proteins are recruited to DNA. Toward that end, one major project in the lab has been to determine all sequences and DNA-binding proteins required for PRE activity. In the Drosophila genome, there are hundreds of PREs that regulate a similar number of genes, and it was not known whether all PREs are alike. Our recent data showed that there is functional and architectural diversity among PREs, suggesting that PREs adapt to the environment of the gene they regulate. PREs are made up of binding sites for several DNA-binding proteins. Over the years, our lab identified Pho, Pho-like, Spps, and Combgap as DNA proteins that bind to PREs. Our recent genome-wide studies show that different PREs require distinct DNA-binding proteins. In addition, our work illustrates the combinatorial nature and redundancy of PcG recruitment in Drosophila.

A second major project in the lab is to determine how the PREs of the engrailed/invected (en/inv) gene complex control these genes in their native location. Surprisingly, we found that not all PREs are required in vivo, suggesting a redundancy in PRE function. To understand the interplay between PREs and enhancers (sequences important for activation of gene expression), we completed an analysis of the regulatory DNA of the en/inv gene complex. We found that regulatory sequences are spread throughout a region of at least 79kb in that gene complex and that the same enhancers activate both engrailed and invected expression. In addition, we showed that a 79-kb transgene (HA-en79), which contains the en gene and flanking regulatory DNA, is able to rescue a deletion for the entire inv-en locus. Our current studies explore the effects of the chromosomal neighborhood on gene
Polycomb group response elements (PREs)

PcG proteins act in protein complexes that repress gene expression by modifying chromatin [Reference 1]. The best-studied PcG protein complexes are PRC1 and PRC2. PRC2 contains the histone methyltransferase Enhancer of Zeste, which tri-methylates lysine 27 on histone H3 (H3K27me3). The chromatin mark H3K27me3 is the signature of PRC2 function. At most well-studied genes, PRC2 acts with PRC1, which binds to H3K27me3 and inhibits chromatin remodeling. In Drosophila, PRC1 and PRC2 are recruited to the DNA by PREs. We are interested in determining how this occurs, and, to that end, we defined all the DNA sequences and are finding all DNA–binding proteins required for the activity of a single 181-bp PRE of the Drosophila engrailed gene (PRE2). We found that binding sites for seven different proteins are required for the activity of the PRE2 (Figure 1). There are several binding sites for some of these proteins. Different PREs have different architectures (Figure 1). Our laboratory identified four PRE DNA–binding proteins: Pho, Pho1, Spps, and, most recently, Combgap. The Combgap protein has 10 zinc fingers and recognizes the sequence GTGTGT.

PRE activity can be studied in transgenes, where a single PRE can recruit PcG protein complexes and silence the expression of a reporter gene. In transgenes, mutation of the Spps, Pho, or Combgap binding sites within the PRE obliterates its ability to recruit PcG proteins, and the reporter gene is expressed. Thus, transcriptional silencing by a single PRE in a transgene requires the combinatorial activity of many DNA–binding proteins. We were interested to determine what happens when one of the PRE DNA–binding proteins (the ‘recruiters’) from the genome is removed, and we examined the effect on PcG recruitment genome-
FIGURE 2. Model of the *inv-en* PcG domain in the transcriptionally silenced state

The *inv* and *en* genes are covered with H3K27me3 and are transcriptionally silent. PcG proteins are associated with this domain. There are strong, constitutive PREs, as well as ‘weak,’ tissue-specific PREs. ‘Weak’ PREs often overlap enhancers and are active in some tissues but inactive in others. Actively transcribed genes remain segregated from the PcG domain and determine the limits of the PcG domain (reprinted from Reference 3).

wide. We studied PcG binding genome-wide in mutants that lack the recruiters Spps or Pho [Reference 2]. We found that PcG recruitment to some PREs was completely disrupted, whereas recruitment of PcG proteins was hardly diminished at most PREs. Most PcG domains, which are covered by the chromatin mark H3K27me3, contain several PREs. We believe that the structure of the H3K27me3 domains stabilizes genomic PREs to the loss of one recruiter. However, there are different kinds of PREs, and some are uniquely sensitive to the loss of one recruiter. Our study highlights the complexity and diversity of PcG recruitment mechanisms.

In addition to identifying hundreds of presumptive PREs within Polycomb (H3K27me3) domains, there are thousands of presumptive PREs outside the H3K27me3 domains in larvae [Reference 2]. What is the function of these PREs? Our data suggest that many are tissue-specific PREs, given that H3K27me3 is detected in these locations in some cell types. However, at other locations, no H3K27me3 is detected in any cell type. Our on-going studies are addressing the interesting question as to whether these are PREs and to the nature of their function.

The role of PREs at the *en* gene

The *Drosophila engrailed* (*en*) gene encodes a homeodomain protein that plays an important role in the development of many parts of the embryo, including formation of the segments, nervous system, head, and gut. By specifying the posterior compartment of each imaginal disc, *en* also plays a significant role in the development of the adult. Accordingly, *en* is expressed in a highly specific and complex manner in the developing organism. The *en* gene exists in a gene complex with *inverted* (*inv*), an adjacent gene; *inv* encodes a protein with a nearly identical homeodomain; *en* and *inv* are co-regulated and express proteins with largely redundant functions. Unlike *en*, *inv* is dispensable for *Drosophila* viability in the laboratory.

The *en* and *inv* genes exist in a 113kb domain that is covered by the H3K27me3 chromatin mark (Figure 2). Within the *en/inv* domain there are four major PREs, strong peaks of PcG protein binding. One popular model posits that DNA-binding proteins bound to the PREs recruit PcG protein complexes and that PRC2 tri-methylates histone H3 throughout the domain until PRC2 comes to either an insulator or an actively
transcribed gene. There are two PREs upstream of the en transcription unit, PRE1 and PRE2 (Figure 1). Both PREs reside within a 1.5kb fragment located from –1.9kb to –400bp upstream of the major en transcription start site. There are also two major inv PREs, one located at the promoter and another about 6kb upstream of that. Our laboratory showed that all these PREs have the functional properties attributed to PREs in transgenic assays. To test their function at the intact en-inv domain, we set out to delete these PREs from the genome. Given that PREs work as repressive elements, the predicted phenotype of a PRE deletion is a gain-of-function ectopic expression phenotype. Unexpectedly, when we made a 1.5kb deletion removing PRE1 and PRE2, flies were viable and had a partial loss-of-function phenotype in the wing. Similarly, deletion of inv PREs yielded viable flies with no mis-expression of en or inv. Importantly, the H3K27me3 en/inv domain is not disrupted in either of these mutants [Reference 3].

In Drosophila, PREs are easily recognizable in chromatin immunoprecipitation experiments as discrete peaks of PcG protein binding, but the H3K27me3 mark spreads throughout large regions. PcG proteins are conserved in mammals; however, PcG binding usually does not occur in sharp peaks, and PREs have been much harder to identify. We created a chromosome in which both the en and inv PREs are deleted [Reference 3]. Surprisingly, the flies are viable, and there is no mis-expression of en or inv in embryos or larvae. The question arises as to how PcG proteins are recruited to the inv-en domain in the absence of these PREs. We performed chromatin-immunoprecipitation followed by Next-Gen sequencing (ChIP-seq) on the PcG proteins Pho and Polyhomeotic (Ph). The data showed that, in addition to the large Pho/Ph peaks at the known PREs, there are many smaller Pho/Ph peaks within the inv/en domain. We found that those peaks may also function as PREs. Thus, rather than a few PREs, there are many PREs controlling inv-en expression, and some may act in tissue-specific ways [Reference 3]. Our work shows that there are two types of PREs in Drosophila: strong, constitutive PREs and tissue-specific PREs that tend to overlap with enhancers (Figure 2).

The inv-en gene complex is flanked by tou and E(Pc), two ubiquitously expressed genes (Figures 2 & 3). The H3K27me3 mark stops at the two genes. We believe that it is the transcription of these two genes that blocks the spreading of the H3K27me3 mark and stabilizes the repression of inv and en by PcG proteins. To test this assumption, we made a large transgene marked by HA–tagged Engrailed protein. A 79-kb HA-en transgene was able to correctly express En and completely rescue inv-en double mutants. We inserted the transgene into
FIGURE 4. Enhancers of the \textit{invected} and \textit{engrailed} genes

\textbf{A.} P-element vector (P[\textit{en}]), used to assay the function of \textit{en}–regulatory DNA, contains the \textit{en} promoter, 396bp of upstream sequences, and an untranslated leader fusion between the \textit{en} transcript and the \textit{Adh-lacZ} reporter gene. \textit{inv/en} DNA fragments were added to this vector at the location of the triangle.

\textbf{B.} The extent of each fragment cloned into \textit{P[en]} is shown as a black line with a letter above the \textit{inv/en} genomic DNA map (indicated by a long black line with hatch marks at 10kb intervals; numbers are coordinates on chromosome 2R, genome release v5). Expression pattern in embryos or the wing imaginal disc (wd) are shown above or below the genomic DNA, with arrows pointing to the fragment(s) that generate(s) the pattern. Figure reprinted from Cheng Y \textit{et al.} \textit{Dev Biol} 2014;395:131.

other places in the \textit{Drosophila} genome [Reference 4]. Our data showed that, while the information to form the H3K27me3 domain is contained within the 79-kb HA-en transgene, the structure of the H3K27me3 domains differs from that at the endogenous locus. Specifically, the H3K27me3 mark spread beyond the transgene into flanking DNA. Further, enhancers within the 79-kb HA-en transgene could interact with some flanking genes and drive their expression in subsets of the En pattern. Also, removal of the PREs from the transgene led to loss of PcG silencing in the abdominal segments of the flies. These data provide evidence that the endogenous \textit{inv-en} domain imparts stability to the locus and facilitates both transcriptional activation and silencing of these two developmentally important genes.

\textbf{Enhancer-promoter communication}

Enhancers are often located tens or even hundreds of kb away from their promoter, sometimes even closer to the promoters of genes other than the one they activate. Several years ago, we showed that \textit{en} enhancers can act over large distances, even skipping over other transcription units, choosing the \textit{en} promoter over those of neighboring genes. Such specificity is achieved in at least three ways. First, early-acting enhancers that drive \textit{engrailed} expression in stripes exhibit promoter specificity. Second, a proximal promoter-tethering element is required for the action of the imaginal disc enhancer(s). Our data point to two partially redundant promoter-tethering elements. Third, the long-distance action of \textit{en} enhancers requires a combination of the \textit{en} promoter and sequences within or closely linked to the promoter-proximal PREs. The data show that several mechanisms ensure proper enhancer-promoter specificity at the \textit{Drosophila en} locus, providing one of the first detailed views of how promoter-enhancer specificity is achieved.

As a follow-up to these studies, we located all the enhancers that regulate the transcription of \textit{en} and the closely linked co-regulated \textit{inv} gene (Figure 4). Our dissection of \textit{inv-en}–regulatory DNA showed that most enhancers are spread throughout a 62kb region. We used two types of constructs to analyze the function of
this DNA: P-element–based reporter constructs with small pieces of DNA fused to the en promoter driving lacZ expression (Figure 4); and large constructs with HA–tagged en and inv inserted in the genome with the phiC31 integrase. In addition, we generated deletions of inv and en DNA in situ and assayed their effects on inv/en expression. Our results support and extend our knowledge of inv-en regulation. First, inv and en share regulatory DNA, most of which flanks the en transcription unit. In support of this finding, a 79-kb HA-en transgene can rescue inv en double mutants into viable, fertile adults. In contrast, an 84-kb HA-inv transgene lacks most of the enhancers for inv and en expression. Second, there are several enhancers for inv/en stripes in embryos; some may be redundant, but others play discrete roles at different stages of embryonic development. Finally, no small reporter construct gave expression in the posterior compartment of imaginal discs, a hallmark of inv/en expression. Robust expression of HA-en in the posterior compartment of imaginal discs is evident from the 79-kb HA-en transgene, while a 45-kb HA-en transgene gives weaker, variable imaginal disc expression. We suggest that the activity of the imaginal disc enhancer(s) depends on the chromatin structure of the inv-en domain. We are currently investigating the properties of the inv/en imaginal disc enhancer(s) using a variety of methods, including deleting them from the endogenous inv/en domain using CRISPR/cas9.

**Defining the ends of Polycomb domains in Drosophila**

Actively transcribed genes flank many Polycomb domains, and previous genomic studies showed that inhibition of transcription using chemical inhibitors leads to a spreading of H3K27me3 in the genome. We conducted a genome-wide analysis of Polycomb boundaries in Drosophila larvae [Reference 5]. We found six different types of Polycomb-domain boundaries, including those made by insulator proteins, and actively transcribed genes. The inv-en Polycomb domain is flanked by two actively transcribed genes, E(Pc) and tou (Figure 3). Insertion of a transcriptional stop within the tou gene causes an extension of the H3K27me3 mark to the point of active transcription. We also suggest that active transcription limits the range of inv-en enhancers and that promoter specificity is important for inv-en enhancer activity [Reference 5]. Currently, we are working to add actively transcribed genes, as well as, separately, to add insulators to delimit the ends of the 79-kb transgene. We hypothesize that adding boundaries will increase the accuracy and robustness of the expression of the transgene.

**Publications**

Collaborators

- Karl Pfeifer, PhD, Section on Epigenetics, NICHD, Bethesda, MD

Contact

For more information, email jkassis@mail.nih.gov or visit http://kassislab.nichd.nih.gov.
Extracellular Matrix Disorders: Molecular Mechanisms and Treatment Targets

The extracellular matrix (ECM) is responsible for the structural integrity of tissues and organs as well as for maintaining an optimal environment for cellular function. ECM pathology is involved in a wide variety of disorders, ranging from rare genetic abnormalities of skeletal development (skeletal dysplasias) to such common ailments as osteoporosis, fibrosis, and cancer. Collagens are triple-helical proteins that form the structural scaffolds of the ECM. Their procollagen precursors are assembled and folded from three pro-alpha chains in the endoplasmic reticulum (ER), trafficked through the Golgi apparatus, secreted, and then converted into mature collagen by enzymatic cleavage of propeptides. The most common collagen is type I, which is a heterotrimer of two pro-alpha1(I) chains and one pro-alpha2(I) chain and is by far the most abundant protein in all vertebrates. Type I collagen fibers form the organic scaffold of bone, tendons, ligaments, and the matrix of skin and many other tissues. We focus on translational studies of developmental disorders of the ECM such as osteogenesis imperfecta (OI), Ehlers-Danlos syndrome (EDS), and chondrodysplasias, as well as related ECM pathologies in fibrosis, cancer, and osteoporosis. Our goal is to understand molecular mechanisms and thus identify treatment targets in ECM disorders, particularly those involving abnormal metabolism of type I collagen, and to bring this knowledge to clinical research and practice.

Procollagen folding and its role in ECM disorders

Osteoblasts and fibroblasts produce and secrete the massive amounts of type I procollagen needed to build the skeleton and other tissues. Type I procollagen is one of the most difficult proteins to fold. Its massive production presents a unique challenge for protein quality control and trafficking. We discovered that, above 35°C, the conformation of natively folded human procollagen is less favorable than the unfolded one. To fold procollagen at body temperature, cells use specialized ER chaperones to stabilize the native conformation. Outside the cell, the native conformation is stabilized after procollagen is converted to collagen and incorporated into collagen fibers. Unincorporated molecules denature within several hours of secretion and become susceptible to rapid proteolytic degradation. Up to 10–15% of procollagen is misfolded even under normal conditions,
necessitating activation of cell stress–response pathways that are responsible for degradation of misfolded molecules and which force the cell to always function in a high-stress mode. Our findings indicate that one of the key pathophysiological mechanisms of OI and other hereditary type I collagen–metabolism disorders is cell stress caused by excessive procollagen misfolding, inability of the cell to handle the normal load of misfolded procollagen, or both.

While we focus mostly on hereditary disorders affecting bone and other tissue development in children, excessive procollagen misfolding of nonhereditary origin is also likely. Our data suggest that such misfolding should occur upon changes in the ER associated with environmental factors, inflammation, aging, etc. It is likely to contribute to fibrosis, cancer, age-related osteoporosis, and many other common ailments. However, little is known about procollagen misfolding pathophysiology because underlying molecular mechanisms and consequences of excessive misfolding for the cell remain poorly understood.

The most common hereditary cause of procollagen misfolding is a Gly substitution anywhere in the obligatory (Gly-X-Y)n sequence that distinguishes all collagens. Such substitutions in type I collagen are responsible for over 80% of severe OI cases. Similar substitutions in other collagens cause EDS and a variety of other syndromes. Their pathophysiology is one of the key topics of our studies. For instance, our studies on OI patients with over 50 different Gly substitutions revealed several structural regions within the collagen where these mutations might be responsible for distinct OI phenotypes. One such region is the first 85–90 amino acids at the N-terminal end of the triple helix, mutations in which prevent normal N-propeptide cleavage. Incorporation of the uncleaved molecules into collagen fibrils leads to distinct OI/EDS with hyperextensibility and joint laxity.

Cell biology of procollagen misfolding

Our current research focuses on the cell biology of procollagen misfolding. In one approach, we use live-cell imaging to investigate the synthesis, folding, trafficking, and degradation of fluorescently tagged procollagen in osteoblasts. Such imaging of osteoblasts transiently transfected with fluorescent procollagen chains revealed new pathways of sorting and trafficking of normally folded and misfolded procollagen molecules in the cell. As expected, normally folded procollagen is loaded into Golgi-bound transport vesicles at ER exit sites (ERES) that are marked by the coat protein complex II (COPII). Contrary to widely held beliefs, however, the vesicles do not have a COPII coat nor do they contain HSP47, a collagen-specific ER chaperone that preferentially binds to natively folded procollagen to assist in its folding and loading into ERES. Instead, transport vesicle formation appears to be dependent on COPI coat assembly and HSP47 release at distal regions of ERES, potentially explaining unusual features of bone pathology caused by mutations in HSP47 and COPI coat proteins. Misfolded procollagen is retained at ERES, resulting in a COPII–dependent modification of ERES membranes by ubiquitin and autophagic machinery. We discovered that such ERESs are then directly engulfed by lysosomes and degraded, delineating a new ERES micro-autophagy pathway. We are currently investigating whether mutations in the COPII protein SEC24D cause bone pathology by disrupting this pathway.

Rerouting of ERES–loaded cargo from secretion to micro-autophagy may have wide implications. It is likely to be a general rather than collagen-specific phenomenon, considering the known COPII coat involvement in both protein secretion and degradation. The hypothesis is currently under investigation in our and several collaborating laboratories. From clinical and translational perspectives, our findings may explain why patients with mutations in different COPII proteins have different pathologies in the development of bone, cartilage, and other tissues.
To gain all these and additional insights, we are expanding the toolbox for studies of collagen metabolism pathology by exploiting emerging gene-editing technologies. We created an osteoblast cell line in which the endogenous pro-alpha2(I) chain has a fluorescent tag and Flp–recombinase target sites for replacing the tag with other peptides. We demonstrated that the cells produce and mineralize bone-like ECM, enabling us to perform live-cell imaging of endogenous procollagen, confirming its trafficking pathways. Given the interest of other researchers in using such cells for a variety of mechanistic studies, we are currently in the process of depositing several clones for public distribution. We also believe that the same strategy can then be used to generate mouse models and study other proteins.

In another approach, we are investigating the pathophysiology of cell-stress response to procollagen misfolding caused by a Gly610 to Cys substitution in the triple-helical region of pro-alpha2(I). We helped develop a mouse model of OI with this mutation (G610C mouse), which mimics the pathology found in a large group of patients with the same mutation. Our study of this model revealed misfolding and accumulation of mutant procollagen in the ER of fibroblasts and osteoblasts, resulting in cell stress and malfunction. We are therefore investigating the mechanism this stress and its role in pathology by altering how the cells adapt to it and by examining cell-stress response pathways activated by the mutation. First, we found that reduced autophagic degradation of misfolded procollagen resulting from reduced expression of an essential autophagy protein ATG5 causes about 40% perinatal lethality of the animals, apparently owing to malfunction of lung fibroblasts. Since lung pathology is the most common cause of death in OI patients, we are examining the underlying molecular mechanisms and potential targets for therapeutic intervention. Second, we demonstrated that knockout of ATG5 in mature osteoblasts increased bone pathology, confirming the pathogenicity of misfolded procollagen accumulation in osteoblast ER in vivo and the role of autophagy in reducing such accumulation. Third, our ongoing single-cell RNA studies are demonstrating that the G610C mutation activates a previously unknown, noncanonical cell-stress response: increased transcription of cell-stress transducer genes \textit{Atf5} and \textit{Hspa9} instead of their homologs \textit{Atf4} and \textit{Hspa5} that are known to be involved in unfolded protein response pathways of canonical ER stress. We confirmed this noncanonical cell-stress response by \textit{in situ} RNA hybridization, identified several candidate pathways involved, and are currently examining the underlying molecular mechanisms.

New approaches to analysis and treatment of ECM pathology
Our observations suggested that the pathology associated with procollagen misfolding may be partially reversed by improving cell adaptation to misfolded procollagen accumulation in the ER, thereby improving lung fibroblast and osteoblast function. Although this would not eliminate the detrimental effects of secreted mutant collagen, pharmacological treatment of cell malfunction is the most realistic short-term strategy for targeting the causes rather than the effect of bone pathology in OI. It is also likely to be a good long-term strategy for the treatment of cell malfunction caused by procollagen misfolding in cases that do not involve pathogenic mutations.

To pursue the strategy, we are examining the effects of enhancing the natural ability of cells to remove and degrade misfolded molecules via autophagy, which is the simplest way to prevent their pathogenic accumulation in the ER. Our preliminary study of autophagy enhancement by a low-protein diet (LPD) in G610C mice revealed improved osteoblast differentiation and function, resulting in better bone quality, but prolonged LPD stunted animal growth. We are thus testing intermittent LPD and fasting approaches that might provide the same benefits of autophagy enhancement without long-term nutrient deficiency.
At the same time, we are validating the general approach of autophagy enhancement by direct genetic modulation of autophagy efficiency. For instance, our studies on the effects of reduced and increased ATG5 expression confirmed that osteoblasts also degrade misfolded procollagen primarily by ERES micro-autophagy in vivo. We found that this autophagy pathway is only moderately affected by ATG5 expression, necessitating a search for other therapeutic targets. We are therefore testing drugs known to reduce the accumulation of misfolded proteins in the ER by enhancing their secretion and autophagy (e.g., 4-phenylbutyrate or 4PBA) and drugs known to reduce the impact of this accumulation on protein synthesis (e.g., ISRIB). We found that 4PBA reduces bone pathology in a zebrafish model of OI and in G610C mice, but that 4PBA is very rapidly metabolized and therefore difficult to deliver to the targeted tissues in the proper therapeutic dosage. Our studies of ISRIB and other drugs are still in exploratory stages.

A key issue in monitoring treatment efficiency in animal models as well as in general diagnostic analysis of bone pathology is the lack of reliable methods with which to characterize the function of bone cells. Traditional histopathology relies on subjective analysis of bone-cell morphology in tissue sections, which is not a reliable indicator of cell function. Over the last two years, we developed a new approach for visualizing and quantifying mRNA expression in individual cells in bone sections. The approach enables objective and reliable cell identification as well as in situ characterization of cell differentiation and function. Based on the interest of bone histomorphometry experts in learning this approach, we hope that it will soon be adapted not only for research but also for clinical practice.

**Translational studies on patients with novel or unusual forms of skeletal dysplasia**

Over the last several years, we assisted several clinical research groups in characterizing collagen metabolism pathology in cells from patients with newly discovered skeletal dysplasias caused by mutations in cartilage-associated protein (CRTAP), prolyl-3-hydroxylase (P3H1), cyclophilin B (CYPB), the collagen-binding molecular chaperone FKBP65, the signaling protein WNT1, the ER–membrane ion channel TRICB, Golgi-membrane metalloprotease S2P, the transmembrane anterior-posterior transformation protein 1 (TAPT1), or collagen prolyl-4-hydroxylase 1 (P4H1). Our studies suggested that the CRTAP/P3H1/CYPB complex functions as a procollagen chaperone. A deficiency in any of the three proteins delays procollagen folding, although their exact roles remain unclear. More surprisingly, we found no detectable changes in the procollagen folding rate in cultured fibroblasts from patients with FKBP65 mutations. Our data suggested that FKBP65 may affect post-translational modification of procollagen and deposition of collagen matrix by a different mechanism. It remains unclear why some FKBP65 mutations cause severe OI with joint contractures (Bruck's disease), while others cause joint contractures without pronounced OI (Kuskokwim syndrome) or OI without pronounced joint contractures. Our study of TRICB–deficient cells revealed abnormal conformation and reduced thermal stability of type I procollagen, suggesting dysregulation of collagen chaperones in the ER or direct involvement of TRICB in procollagen folding. Our experiments indicated that the pathogenic effects of mutations in the transmembrane protein TAPT1 and in site-2 metalloprotease (S2P) might not be directly related to disruptions in synthesis, folding, or trafficking of procollagen chains. As expected, we found that patient cells with mutant P4H1 secreted abnormal procollagen that had significantly reduced thermal stability owing to under-hydroxylation of proline residues by P4H1. Surprisingly, however, we found no abnormalities in the procollagen folding or secretion rates, no evidence of misfolded procollagen accumulation in the cell, and no evidence of altered ER chaperone composition.
We also continued translational studies of OI caused by missense mutations in type I collagen that are not substitutions of obligatory Gly residues, specifically focusing on substitutions of Y-position arginine (Y-Arg) residues in the Gly-X-Y triplets within the collagen triple helix. We found that Y-Arg substitutions cause procollagen misfolding and accumulation in the ER to almost the same extent as Gly substitutions, because Y-Arg enhances collagen triple-helix stability and promotes triple-helix folding through binding of HSP47.

Presently, we are examining the molecular mechanism underlying OI caused by mutations in the ER-membrane stress receptor CREB3L1/OASIS. Preliminary analysis of RNA-seq and qPCR data combined with our new mRNA-based histopathology assay suggests common regulation CREB3L1 and some COPII proteins involved in secretory and/or autophagic procollagen export from the ER. However, the study is still in an early phase, and much work remains to be done.

**Extracellular matrix pathology in tumors and fibrosis**

Another aspect of our collagen metabolism pathology studies has been characterization of the pathology in fibromas and tumors, e.g., abnormal collagen composition of uterine fibroids and the potential role of type I collagen homotrimers in cancer. The normal isoform of type I collagen is a heterotrimer of two alpha1(I) chains and one alpha2(I) chain. Homotrimers of three alpha1(I) chains are produced in some fetal tissues, carcinomas, fibrotic tissues, as well as in rare forms of OI and EDS associated with alpha2(I) chain deficiency. We found the homotrimers to be at least 5–10 times more resistant to cleavage by all mammalian collagenases than the heterotrimers, and we determined the molecular mechanism of this resistance. Our studies suggested that cancer cells might utilize the collagen isoform to build collagenase-resistant tracks, thus supporting invasion through stroma of lower resistance.

We also investigated bone pathology and tumors caused by defects in cAMP signaling, e.g., associated with mutation in protein kinase A (PKA), which is a key enzyme in the cAMP signaling pathway. Initially, we studied synthesis of type I collagen homotrimers. However, over the last 3–5 years, our focus has shifted to abnormal differentiation of osteoblastic cells and deposition of bone. We found that knockouts of various PKA subunits cause not only abnormal organization and mineralization of bone matrix but also novel bone structures that had not been previously reported. For instance, we observed free-standing cylindrical bone spicules with an osteon-like organization of lamellae and osteocytes but an inverted mineralization pattern, a highly mineralized central core, and diminishing mineralization away from the central core. We assisted clinical researchers in characterizing abnormal osteoblast maturation, the role of an abnormal inflammatory response, and effects of anti-inflammatory drug treatments in such animals. Improved understanding of bone tumors caused by PKA deficiencies may not only clarify the role of cAMP signaling but may also suggest new approaches to therapeutic manipulation of bone formation in skeletal dysplasias.

**Multimodal micro-spectroscopic imaging and mapping of tissues**

Given that tissue analysis plays a crucial role in understanding and treating collagen metabolism disorders, we are developing methods to characterize not only cell function in tissue sections but also ECM composition and structure. Label-free micro-spectroscopic infrared and Raman imaging of tissues and cell cultures provides important information about the chemical composition, organization, and biological reactions inaccessible by traditional histology. By resolving the problem of light-path variations with passive thermomechanical stabilization, we developed high-definition (HD) infrared imaging and Raman micro-spectroscopic methods, achieving spectral reproducibility of up to two orders of magnitude better than with leading commercial
instruments. The HD technology was essential for the analysis of abnormal collagen matrix deposition by CRTAP– and FKBP65–deficient cells. It has enabled us to assist NIBIB scientists in characterizing a functionalized carbon-nanotube approach to the delivery of anticancer agents into cells that overexpress hyaluronate receptors and is crucial to our current studies of bone structure and mineralization in the mouse models of OI and PKA deficiencies described above.

The power of the technology is illustrated by our studies of ECM structure and composition in a mouse model of diastrophic dysplasia (DTD). DTD is an autosomal recessive dysplasia that affects cartilage and bone development and is caused by mutations in the SLC26A2 sulfate transporter gene, deficient sulfate uptake by chondrocytes, and resulting under-sulfation of glycosaminoglycans in cartilage matrix. For instance, we found that chondroitin under-sulfation leads to disorientation of collagen fibers, disrupting a thin protective layer at the articular surface and causing subsequent cartilage degradation. We also investigated the relationship between chondroitin under-sulfation and the rate of its synthesis across the growing epiphyseal cartilage and built a mathematical model for the sulfation pathway, predicting treatment targets for sulfation-related chondrodysplasias and genes that might contribute to the juvenile idiopathic arthritis recently associated with single-nucleotide polymorphisms in SLC26A2. We are further extending the technology by combining imaging of bone and cartilage ECM composition and structure with biomechanical measurements at the same length scales and in vivo ECM studies at large scales by solid-state magnetic resonance imaging (MRI) being developed by our collaborators.

As a test of the technology and its important translational application, we are presently working on combining our advances in mRNA-based and micro-spectroscopy-based histopathological analysis for understanding normal growth plate homeostasis and growth plate pathology in OI. General growth deficiency and disproportional development of proximal and distal limb bones (rhizomelia) are common, clinically important, and yet poorly understood features of the disease. Spatially resolved imaging of mRNA at a single-cell level is enabling us to identify the progression of growth-plate chondrocytes through differentiation steps and expression of different ECM components at these steps. Spatially resolved micro-spectroscopic analysis of ECM organization at the same distance scales in the same tissue sections is enabling us to relate the cell differentiation and function to ECM composition, structure, and function.

**Publications**

4. Omari S, Makareeva E, Gorrell L, Jarnik M, Lippincott-Schwartz J, Leikin S. Mechanisms of procollagen and


**Collaborators**

- Peter Basser, PhD, *Section on Quantitative Imaging and Tissue Sciences, NICHD, Bethesda, MD*
- Carsten Bönnemann, MD, *Neuromuscular and Neurogenetic Disorders of Childhood Section, NINDS, Bethesda, MD*
- Peter H. Byers, MD, *University of Washington, Seattle, WA*
- Antonella Forlino, PhD, *Università degli Studi di Pavia, Pavia, Italy*
- Ken Kozloff, PhD, *University of Michigan, Ann Arbor, MI*
- Jennifer A. Lippincott-Schwartz, PhD, *Howard Hughes Medical Institute, Janelia Research Campus, Ashburn, VA, and Adjunct Investigator, NICHD, Bethesda, MD*
- Fransiska Malfait, MD, PhD, *Center for Medical Genetics, Ghent, Belgium*
- Joan C. Marini, MD, PhD, *Section on Heritable Disorders of Bone and Extracellular Matrix, NICHD, Bethesda, MD*
- Pamela G. Robey, PhD, *Craniofacial and Skeletal Diseases Branch, NIDCR, Bethesda, MD*
- Antonio Rossi, PhD, *Università degli Studi di Pavia, Pavia, Italy*
- Constantine A. Stratakis, MD, D(med)Sci, *Section on Endocrinology and Genetics, NICHD, Bethesda, MD*
- Sofie Symoens, PhD, *Center for Medical Genetics, Ghent, Belgium*

**Contact**

For more information, email leikins@mail.nih.gov or visit http://physbiochem.nichd.nih.gov.
From Axon Damage to Disease: Common Pathways in Neurodegeneration

Our work is dedicated to a better understanding of common molecular and cellular mechanisms of neurodegeneration, with the ultimate goal of developing treatments for neurodegenerative diseases and even preventing them. The lab currently focuses on an evolutionarily conserved neuronal stress-response pathway under the control of DLK (dual leucine zipper kinase), which plays an important role in several neuropathologies. As a cellular stress-response pathway in neurons, its function is to promote recovery from injury; however, at the same time, it can drive several types of pathologies, including peripheral neuropathies and neurodegeneration.

The hypothesis driving our work is that common mechanisms are responsible for neurodegeneration during development, childhood, and aging. Most of what is currently understood about neurodegenerative disease stems from the identification of genetic linkages that are causative or predisposing, and from efforts to uncover the mechanisms underlying such linkages. However, the linkages account for only a relatively small proportion of all cases. The vast majority of cases have no established genetic etiology and therefore no clear pathway to target. An understanding of any common mechanisms involved in neurodegeneration would provide major breakthroughs for designing treatments. We showed that dual leucine zipper kinase (DLK, also known as MAP3K12) acts as a crucial downstream node in neurodegeneration and neuropathy, two pathologies with very different causes and outcomes [References 3 & 4]. The lab is currently investigating how such diverse diseases converge upon this single pathway and how this pathway mediates divergent fates.

Motor neuron identity in health and disease

Our most recent publication concerns collaborative work with the lab of Nick Ryba, in which we perform single-nucleus RNA sequencing of sensory neurons with or without nerve injury, and examine neuron fate on an individual cell basis [Reference 2]. We found that nerve injury induces profound transcriptional reprogramming in sensory neurons that normally exist in many different types and that, upon injury, lose their original identity and switch to a completely novel, injured neuronal identity. We also found that sensory neurons have the potential to recover, returning to their native transcriptional state.
In parallel work, the lab is now investigating whether the same holds true for skeletal motor neurons, and if not, in what ways such neurons differ in their injury response. The work is relevant to motor neuron diseases of many kinds, in which it is thought that specific subtypes are more vulnerable to disease than others. We began with a description of the motor neuron types that exist, given that, although the existence of many subtypes was known, their diversity overall and the best markers were not known. This year, we produced an atlas of cholinergic neurons of the adult mouse spinal cord [Reference 1]. The work is currently in revision for Nature Communications. We will follow up by studying the disease state and investigating whether motor neurons undergo transcriptional reprogramming similar to the sensory neurons after nerve injury and what happens to these cells over the long term, after acute nerve injuries and also in disease models.

Using the neuronal injury response to study neurodegeneration in the mammalian CNS

The existence of common mechanisms of neurodegeneration has long been hypothesized. My previous work focused on DLK, a MAP3 kinase (mitogen activated protein triple kinase) that had been known, upon injury, to initiate a retrograde stress-signaling cascade from the axon to the cell body. The work uncovered an important role for DLK signaling in promoting neuronal death as well as controlling synapse density, using several different animal models of neurodegeneration. We also showed that human disease tissue bears markers of DLK/JNK signaling activation [Reference 4]. The most exciting implication of this study is that DLK is part of a long-sought common mechanism of neurodegeneration, which has led to its becoming a promising drug target for the treatment of several diseases.

In new work, we generated a reporter mouse line that we named Stress-TRAP, which expresses Cre to permanently label neurons that have engaged in injury signaling, so as to visualize, track, enrich for, and study injured neurons in the context of acute injury models (e.g., nerve injury, traumatic brain injury) or neurodegenerative diseases caused by inherited mutations. We will use these mouse lines to perform single-cell sequencing studies, as well as whole cleared brain imaging, in which we selectively labeled the neurons undergoing injury signaling. Especially at early stages of disease, we anticipate the tool will be of great use in detecting the earliest neuronal pathologies, when the affected neurons are still quite sparse.

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Publications


**Collaborators**

- Carsten Bönnemann, MD, *Neuromuscular and Neurogenetic Disorders of Childhood Section, NINDS, Bethesda, MD*
- Alexander Chesler, PhD, *Sensory Cells and Circuits Section, NCCIH, Bethesda, MD*
- Teresa Dunn-Giroux, PhD, *Uniformed Services University of the Health Sciences, Bethesda, MD*
- Tracey Rouault, MD, *Section on Human Iron Metabolism, NICHD, Bethesda, MD*
- Nicholas Ryba, PhD, *Laboratory of Sensory Biology, NIDCR, Bethesda, MD*
- Michael E. Ward, MD, PhD, *Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda, MD*

**Contact**

For more information, email claire.lepichon@nih.gov or visit [http://lepichon.nichd.nih.gov](http://lepichon.nichd.nih.gov).
Mobile genetic elements create genetic diversity, which, through natural selection, promotes adaptation and innovation. A wealth of examples document how the action of transposable elements (TEs) leads to important modifications in morphology, metabolism, and ultimately fitness. Recent work reveals that large gene networks co-regulated by key transcription factors are built on regulatory sequences derived from TEs. Evolution of the networks depends on the dispersal of regulatory elements to genes that, when coexpressed, provide the host with novel functions such as innate immunity or the pregnancy of mammals. The adaptation and innovation produced by mobile elements comes at the cost of gene disruption resulting from indiscriminate insertion and homologous recombination. In each host species a tenuous balance is struck between the mutagenic activity of TEs and the benefits provided by the genetic diversity they produce, a genetic conflict that is affected by many factors, including host mechanisms that silence TEs and the activity of mobile elements, which can be triggered by environmental stress. When the balance between genome defense and TE activity is perturbed, transposition in gonads leads to infertility and, in the case of humans, \textit{de novo} inserts generate disease alleles. The HIV-1 virus is an example of an infectious mobile element that can overwhelm defense mechanisms. Its efficient integration into the genome of immune cells has resulted in over 35 million deaths.

The long terminal repeat (LTR) retrotransposons of yeast provide a unique opportunity to study in real time the biology and impact of TEs in highly characterized model systems. The Ty1, Ty3, and Ty5 elements of \textit{Saccharomyces cerevisiae} each possess unique mechanisms that limit the disruption of important sequences by directing integration to “safe havens” such as heterochromatin and sequences upstream of RNA polymerase III (pol III) promoters. Our studies on the LTR retrotransposon Tf1 of \textit{Schizosaccharomyces pombe} revealed integration behavior that contrasts sharply with that of the Ty elements. Our large datasets of \textit{de novo} integration show that Tf1 integrates principally into the promoters of RNA pol II–transcribed genes. Promoters are clearly not “safe havens,” raising questions about the biological significance of this integration behavior. Several experiments from our lab showed that Tf1 integration alters promoter activity and is well-suited to promote adaptation to environmental stress.
Transposable element insertions in fission yeast drive adaptation to environmental stress.

Cells are regularly challenged by environmental stress, to which rapid and robust responses are critical for survival. To cope with adverse conditions, cells activate transient programs of transcription that alter expression of hundreds to thousands of genes. Pre-wired transcription responses have evolved as the result of frequent exposure to a common set of external stresses. However, it is not clear how cells cope when confronted with environmental shock, defined here as novel stresses or conditions for which existing responses are inadequate to support survival. Although genetic modifications that improve survival can clearly be achieved through single-nucleotide mutations, these are mostly neutral or detrimental, and would rarely allow survival to abrupt changes in environment. Alternatively, TEs are activated by stress and, because they carry regulatory sequence, TEs can readily alter gene expression. Such a possibility is in line with McClintock’s original proposal that TEs provide a means to overcome the threat of environmental stress by reorganizing the genome [McClintock B. Science 1984;226:792]. However, the tenet has not been directly tested. Tf1 provides a unique opportunity to study the role of transposition in cells’ survival in response to changes in environmental conditions. A stress-response enhancer embedded in Tf1 causes integration to induce the expression of adjacent promoters. The prominent clustering of integration in promoters and the influence of the Tf1 enhancer on adjacent genes suggest the intriguing possibility that Tf1 may be wired to provide efficient adaptation to environmental stress.

To test directly whether transposition plays a significant role in adaptation, we passaged populations of cells in medium containing various concentrations of CoCl₂, which generates reactive oxygen species, causes DNA damage, induces apoptosis, and mimics hypoxia. We used cultures...
in which each cell contained one of 41,000 pre-established insertions of Tf1-neo, which we created by overexpression of Tf1 followed by selection for integration events (Figure 1A). Each cell contained a specific insertion that served as a tag, which we used to measure clonal expansion during competitive growth. With high-throughput sequencing of the Tf1-neo tags, we monitored clonal expansion in cultures grown for 80 generations in 0.0 mM, 0.2 mM, or 1.2 mM CoCl$_2$. Three independent passaging experiments were conducted for each CoCl$_2$ concentration. We assessed whether the Tf1 insertions themselves provided a prominent path to improved growth by identifying clones that expanded in each of the three independent passaging experiments. In cultures grown without CoCl$_2$, the bulk of the insertions maintained constant proportions in the cultures. However, large changes occurred in the cultures that contained CoCl$_2$. With 0.2 mM CoCl$_2$, cells with 106 integration positions reproducibly expanded two-fold or more (Figure 1B). The positions accounted for 3.1% of the initial culture and expanded markedly to become 58%, 52%, and 31% (average of 47%) of the final cultures for passaging experiments 1, 2, and 3, respectively. Such substantial percentages of reproducible tags indicate that the Tf1 insertions contributed significantly to improved growth. Importantly, a significant proportion of the competition-enriched positions were adjacent to genes that participate in TOR pathways (signaling pathways that integrate both intracellular and extracellular signals and serve as a central regulators of cell metabolism, growth, proliferation, and survival), indicating that TOR provides resistance to CoCl$_2$. In S. pombe, the TORC1 and TORC2 kinase pathways activate genes involved in cell growth and stress response, respectively. Deletion of genes involved in both TORC1 and TORC2 pathways, together with studies of strains with recreated insertions, demonstrated that Tf1 integration functioned as the major path to adaptation. To test whether TEs mediate adaptation in natural settings, we analyzed the genomes of 57 wild isolates of S. pombe. We found that polymorphic LTR insertions clustered significantly adjacent to genes that contribute to sporulation frequency, heat shock, and osmotic stress. Our analysis thus indicates that Tf elements function in the wild to provide resistance to environmental stress.

Dense transposon integration reveals that essential cleavage and polyadenylation factors promote heterochromatin formation.

In eukaryotes, the assembly of DNA into highly condensed heterochromatin is critical for a broad range of functions related to genome integrity. The methylation of histone H3 on lysine 9 (H3K9me) is central to the formation of heterochromatin by creating binding sites for a range of chromatin proteins important for silencing transposable elements, chromosome segregation, and epigenetic inheritance. Used extensively for this purpose, S. pombe is an excellent model in which to study the molecular mechanisms that generate and regulate heterochromatin. Centromeres, subtelomeres, and the mating-type region are packaged into constitutive heterochromatin, while meiosis genes are silenced by facultative heterochromatin until cells are starved of nitrogen. Importantly, Clr4, the H3K9-specific histone methyltransferase, is recruited to heterochromatin regions by several mechanisms. Constitutive heterochromatin results from RNAi factors that include the Ago1–containing, RNA–induced transcriptional silencing complex (RITS). Facultative heterochromatin at meiosis genes is independent of RNAi and relies on the RNA elimination (i.e., degradation) factors Red1 and Mmi1 and on the nuclear exosome. However, gaps exist in our understanding of how RNA elimination generates heterochromatin. A new approach for identifying gene function is the high-throughput sequencing of integration profiles, also known as Tn-seq, which identifies genes important for growth under selective conditions. Genes necessary to sustain growth under a specific condition do not tolerate insertions in that condition. Tn-seq has been applied to identify pathogenic genes in bacteria. However, we were the first to develop the method for a eukaryote; we developed a method for identifying essential genes in yeast, and others have subsequently applied the strategy to single-cell eukaryotes.
With the goal of identifying novel factors important for heterochromatin, we produced dense profiles of integrations using the Hermes transposable element and a silencing reporter (ura4) positioned in the outer repeats of centromere 1. Inserts that disrupted genes important for heterochromatin activated ura4, and thus the cells were unable to grow when passaged in 5-fluoroorotic acid (FOA) (Figure 2A). Genes with established roles in heterochromatin assembly had significantly fewer insertions in cells with the centromere reporter otr1R::ura4 than in cells lacking the reporter (Figure 2B). The list of candidates consisted of a total of 199 genes and, importantly, 65 are known to be essential for viability. These essential genes were candidates because they tolerated many insertions in their 3’ sequences that reduced heterochromatin but not viability. The high number of essential genes is significant in that most proteins found to be important for heterochromatin are identified in screens of deletion strains that cannot include essential genes. The 199 candidates showed highly significant enrichments for functions in silencing at centromere outer repeats and included all four factors that produce siRNA.

We identified other RNA–processing factors that were not previously linked to heterochromatin structure. Strikingly, four of the RNA–processing candidates form an interaction module of the canonical mRNA polyadenylation factor and the cleavage factor CPF, as predicted from highly homologous proteins in S. cerevisiae. To determine whether polyadenylation and cleavage contribute to heterochromatin structure at the centromere repeats, we focused on the function of Iss1, a subunit of CPF. We generated a C-terminal truncation of Iss1 (Iss1-ΔC) by removing 38 amino acids that, based on the Hermes insertions, were not important for viability. Iss1-ΔC showed no growth restriction on nonselective medium but exhibited a heterochromatin defect, as demonstrated by growth in the absence of uracil and reduced levels of H3K9 dimethylation (H3K9me2) at otr1R::ura4. The results demonstrated that the Hermes screen correctly identified

**FIGURE 2. Dense maps of transposable element integration identify genes important for heterochromatin at centromere repeats.**

A. Single insertions of the transposable element Hermes were generated in cells with WT cen1 and cen1 otr1R::ura4. Cultures were passaged in 5-fluoroorotic acid (FOA) for 5 or 80 generations. Cells with insertions in heterochromatin genes (het1) express ura4 and cannot grow in FOA. After growth on FOA fewer insertions were detected in het genes in cells with cen1 otr1R::ura4.

B. Genes involved in forming centromere heterochromatin such as mit1 and sir2 had fewer inserts in cells with the cen1 otr1R::ura4 (black, dupl. libraries) than cells with WT cen1 (red, dupl. libraries).
Iss1 as important for heterochromatin structure at the \textit{otr1R::ura4} reporter. Interestingly, we found that Iss1 contributes to the heterochromatin of centromere repeats in cells that lack the \textit{otr1R::ura4} reporter but, in this case, the contribution to H3K9me2 was only observed when the RNAi pathway was disabled by deletion of \textit{ago1}. This role at the outer centromere repeats is therefore independent or redundant with RNAi.

We expanded our study of the Iss1-ΔC mutation to evaluate changes in expression and transcription termination genome-wide. RNA-seq data revealed that Iss1-ΔC did not significantly impact canonical transcription termination, but 73 genes were found to have higher expression. Importantly, the genes overlapped significantly with genes upregulated in cells lacking Rrp6, the 3′-5′ exonuclease subunit of the nuclear exosome. As a key subunit of the nuclear exosome, Rrp6 plays an important role in RNA surveillance in the degradation of meiotic transcripts expressed during vegetative growth and the resulting formation of heterochromatin at these genes. The elimination of meiotic mRNAs depends on the RNA–binding protein Mmi1 to bind to the determinant of selective removal (DSR) sequence in order to recruit the exosome. Our co-immunoprecipitation experiments revealed that Iss1 interacted with Rrp6, Mmi1, and the polyA polymerase Pla1, indicating that Iss1 is associated with this network of elimination factors. Significantly, the interaction with Mmi1 was disrupted by the Iss1-ΔC mutation, a mutation that greatly reduced H3K9me2 at meiotic genes. We tested whether Iss1 plays a direct role in the heterochromatin of meiotic genes by performing ChIP-seq of Iss1-FLAG. While a subset of Iss1–bound genes was highly-expressed and was associated with the canonical function of Iss1 in mRNA termination, most Iss1–bound peaks showed a strong correlation with genes regulated by RNA elimination and heterochromatin. Importantly, the iss1-ΔC mutation caused significant increases in the RNA levels of these genes. Taken together, our studies of RNA levels, Iss1 association with chromatin, and H3K9me2 indicate that Iss1 plays a direct role in the formation of heterochromatin at meiotic genes. Our application of Hermes profiles to identify genes important for heterochromatin formation demonstrates the significance of the approach, especially given that we were able to identify large numbers of essential genes, a result that is not obtainable with other screens.

\textbf{Retrotransposon insertions associated with risk of neurologic and psychiatric diseases}

Neurologic and psychiatric disorders affect 25% of the world population. Given the complexity of the mammalian nervous system, the genetic and cellular etiology of such diseases remains largely unclear. Progress in genetic methodology has provided the potential to identify mechanisms that underlie the diseases. One approach that has successfully identified important disease loci is genome-wide association studies (GWAS). However, in the cases of neurologic and major psychiatric disorders, GWAS have identified large numbers of loci, each associated with small increases in risk. Importantly, there is extensive overlap of the loci that contribute to major psychiatric disorders, indicating that related molecular mechanisms may underlie distinct clinical phenotypes.

The single-nucleotide polymorphisms (SNPs) identified by GWAS to have highest disease association. Trait associated SNPs (TASs) are genetic tags identifying a genomic region that contains the causal mutation(s) leading to the disease risk. Limits on the design of GWAS typically prevent such studies from identifying causal gene alleles. Determining causal variants remains the most challenging and rate-limiting, but also the most important, step in defining the genetic architecture of diseases. The vast majority of GWAS TASs lie in intergenic or intronic regions and therefore do not alter coding sequence. For such SNPs to be causal they would likely have regulatory effects on transcription. Structural variants such as rearrangements, copy number
variants, and TE insertions constitute a substantial and disproportionately large fraction of the genetic variants found to alter gene expression.

In humans, the dominant families of TEs are Long INterspersed Element-1 (LINE-1 or L1) and Alu Short Interspersed Elements (SINEs), which are mobilized by L1. TEs alter gene expression particularly easily because they have evolved various sequences that act on enhancers. Given that TEs make up approximately 45% of the human genome, it is not surprising that their regulatory features are abundant sources of tissue-specific promoter activity.

Relatively recent TE insertions can proliferate in the population and become common alleles. The 1000 Genomes Project described genetic variation of diverse human populations by sequencing whole genomes of 2,504 individuals. The extensive survey of genetic variation detected 17,000 polymorphic insertions of TEs, which have the potential to alter gene expression and affect common disease risk. Some TEs have been implicated at disease loci detected by GWAS.

Given the difficulty in identifying genetic variants responsible for neurologic and psychiatric disorders and the regulatory capacity of TEs, we tested whether polymorphic TEs are potential causative variants of such diseases. We analyzed 593 GWAS of neurologic and psychiatric diseases, which in total reported 753 TASs. From the 17,000 polymorphic TEs, we found that 76 were in linkage disequilibrium (LD) with TASs, indicating that the TEs were among the variants with the potential to be causative. We extended our analysis by evaluating each candidate TE for a role in altering expression of proximal genes. In one approach we determined whether polymorphic TEs could disrupt regulatory sequences, as annotated with the epigenomic data of the NIH Roadmap Epigenomics Consortium. Ten of the TE candidates were located in regions of chromatin with active regulatory function in neurologic tissues. We also tested whether the polymorphic TEs were significantly associated with altered expression of proximal genes. By analyzing multi-tissue expression data from GTEx (Genotype-Tissue Expression project), we found that 31 of the TASs linked to TEs were expression quantitative trait loci (eQTLs, loci that seek to identify genetic variants that affect the expression of one or more genes) for adjacent genes showing correlation with altered expression within regions of the brain. These expression data, together with epigenetic and eQTL analyses, indicate that polymorphic TE insertions are important candidates for causing disease risk for Parkinson's, schizophrenia, and amyotrophic lateral sclerosis, on par with other variants at these loci.

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Publications
3. Grech L, Jeffares DC, Sadée CY, Rodríguez-López M, Bitton DA, Hoti M, Biagosch C, Aravani D,


### Collaborators

- Jürg Bähler, PhD, *University College London, London, United Kingdom*
- Shiv Grewel, PhD, *Laboratory of Biochemistry and Molecular Biology, NCI, Bethesda, MD*
- Stephen Hughes, PhD, *Retroviral Replication Laboratory, HIV Drug Resistance Program, NCI, Frederick, MD*
- Mamuka Kvaratskhelia, PhD, *Ohio State University, Columbus, OH*
- Matthew Plumb, BS, *Ohio State University, Columbus, OH*
- Akira Yamashita, PhD, *National Institute for Basic Biology, Okazaki, Japan*

### Contact

For more information, email [henry_levin@nih.gov](mailto:henry_levin@nih.gov) or visit [http://sete.nichd.nih.gov](http://sete.nichd.nih.gov).
Cell Cycle Regulation in Oogenesis

Our long-term goal is to obtain a comprehensive understanding of how metabolic signaling pathways influence oocyte growth, development, and quality. Chromosome mis-segregation during female meiosis is the leading cause of miscarriages and birth defects in humans. Recent evidence suggests that many meiotic errors occur downstream of defects in oocyte growth and/or the hormonal signaling pathways that drive differentiation of the oocyte. Thus, understanding how oocyte development and growth impact meiotic progression is essential to studies in both reproductive biology and medicine. We use the genetically tractable model organism *Drosophila melanogaster* to examine how meiotic progression is instructed by the developmental and metabolic program of the egg.

In mammals, studies on the early stages of oogenesis face serious technical challenges in that entry into the meiotic cycle, meiotic recombination, and the initiation of the highly conserved prophase I arrest all occur during embryogenesis. By contrast, in *Drosophila* these critical events of early oogenesis all take place continuously within the adult female. Easy access to the early stages of oogenesis, coupled with available genetic and molecular genetic tools, makes *Drosophila* an excellent model for studies on the role of metabolism in oocyte development and maintenance.

The GATOR complex: integrating developmental and metabolic signals in oogenesis

The Target of Rapamycin Complex 1 (TORC1) regulates cell growth and metabolism in response to many inputs, including amino acid availability and intracellular energy status. In the presence of sufficient nutrients and appropriate growth signals, the Ragulator and the Rag GTPases (a complex that regulates lysosomal signaling and trafficking) target TORC1 to lysosomal membranes, where TORC1 associates with its activator, the small GTPase Rheb. Once activated, TORC1 is competent to phosphorylate its downstream targets. The Gap Activity Towards Rags (GATOR) complex is an upstream regulator of TORC1 activity.

The GATOR complex consists of two subcomplexes (Figure 1). The GATOR1 complex inhibits TORC1 activity in response to amino acid
FIGURE 1. The GATOR complex regulates TORC1 activity.

The GATOR2 complex opposes the activity of the TORC1 inhibitor GATOR1.

starvation. GATOR1 is a trimeric protein complex consisting of the proteins Nprl2, Nprl3, and Iml1. Evidence from yeast and mammals indicates that the components of the GATOR1 complex function as GTPase–activating proteins (GAP) that inhibit TORC1 activity by inactivating the Rag GTPases. Notably, Nprl2 and Iml1 are tumor suppressor genes, while mutations in Iml1, known as DEPDC5 in mammals, are a leading cause of hereditary epilepsy.

The GATOR2 complex comprises five proteins: Seh1, Sec13, Mio, Wdr24, and Wdr59. Our work, as well as that of others, found that the GATOR2 complex activates TORC1 by opposing the TORC1–inhibitory activity of GATOR1. Intriguingly, computational analysis indicates that Mio and Seh1, as well as several other members of the GATOR2 complex, have structural features consistent with coatomer proteins and membrane-tethering complexes. In line with the structural similarity to proteins that influence membrane curvature, we showed that three components of the GATOR2 complex, Mio, Seh1, and Wdr24, localize to the outer surface of lysosomes, the site of TORC1 regulation. However, how GATOR2 inhibits GATOR1 activity, thus allowing for the robust activation of TORC1, remains unknown. Additionally, the role of the GATOR1 and GATOR2 complexes in both the development and physiology of multicellular animals remains poorly defined. Over the last year, we used molecular, genetic, and cell-biological approaches to define the role of the GATOR complex in the regulation of meiotic progression and genomic stability during oogenesis. Moreover, we used oogenesis as a model system to define a novel mechanism of TORC1 inhibition in response to both amino acid and growth factor restriction.

A unified model of TORC1 regulation: the Rag GTPase regulates the dynamic behavior of TSC downstream of both amino acid and growth factor restriction.

The dysregulation of the metabolic regulator TORC1 contributes to a wide array of human pathologies. Tuberous sclerosis is a rare multi-organ genetic disorder affecting 1 in 6,000 newborns a year. Mutations in components of the tuberous sclerosis complex (TSC) result in the hyperactivation of TORC1, causing the growth of benign tumors in many parts of the body. Although the importance of the TSC in cell metabolism is well established, a detailed understanding of its regulation has remained elusive. We used Drosophila melanogaster and tissue-culture cells to demonstrate that the GATOR2 complex is a novel regulator of TSC. The GATOR1 subcomplex inhibits the activity of TORC1 by preventing the recruitment of the complex to lysosomes, where it encounters its activator Rheb. Specifically, GATOR1 regulates TORC1 localization by serving as a GTPase–activating protein for RagA/B; in their GTP–bound status, RagA/B recruit TORC1 to lysosomes. The GATOR2 complex opposes the activity of GATOR1.
FIGURE 2. Working model for the role of the GATOR complex in the response to meiotic DSBs

A. After ovarian cysts enter meiosis, meiotic DSBs function to activate and/or maintain a GATOR1/TSC–dependent pathway to ensure low TORC1 activity in early prophase of meiosis I. Low TORC1 activity promotes the timely repair of meiotic DSBs. Currently, whether meiotic DSBs directly activate the GATOR1/TSC pathway or an alternative pathway that works in concert with, or in parallel to, GATOR1/TSC is not known.

B. Subsequently, the GATOR2 component Mio is required to attenuate the activity of the GATOR1/TSC pathway, thus allowing for increased TORC1 activity and the growth and development of the oocyte in later stages of oogenesis.

Using fluorescence recovery after photobleaching (FRAP), we determined that knocking out WDR24, one of the subunits of the GATOR2 complex, resulted in the rapid recruitment of the TSC subunits TSC2 and TSC1 to lysosomes in both HeLa cells and in the Drosophila ovary. Furthermore, we demonstrated that the GATOR2 complex regulates TSC2 dynamics by controlling the guanine nucleotide–binding status of the RagA or RagC small GTPases. Specifically, GDP–bound RagA and GTP–bound RagC promote the dynamic recruitment of TSC2 to lysosome. Moreover, by using a photoconvertible protein–tagged TSC2, we determined that the rapid association of TSC2 to lysosomes is accompanied by its rapid dissociation in wdr24−/− cells. Taking together, we provided both in vitro and in vivo evidence to support the model that the GATOR complex regulates the dynamic cycling of the TSC between lysosomes and the cytoplasm (Figure 2).

Currently, there are two working models for the role of TSC in the inhibition of TORC1 activity. The first model posits that TSC lies exclusively downstream of the PI3K-AKT growth-factor signaling pathway, while the second model proposes that TSC is a critical downstream effector of both the growth-factor signaling and amino acid–sensing pathways. Our findings on the function of the GATOR2 complex are consistent with the second model, which implicate different nucleotide states of the Rag GTPase in the recruitment of TORC1 versus TSC to lysosomes in response to amino acid starvation. Moreover, we determined that the Rag GTPase, which was previously thought to exclusively function in amino acid sensing, regulates the recruitment of TSC to lysosomes in response to growth factor restriction. Thus, our data support a model in which both the amino acid–sensing pathway and growth factor–signaling pathway converge on the Rag GTPase to recruit TSC to lysosomes in response to inhibitory signals. Notably, we found that, in both HeLa cells and Drosophila, the Rag GTPase promotes the rapid exchange of TSC between the lysosome and cytosol in response to negative inputs. Moreover, the rate of exchange mirrors TSC function, with depletions of the Rag GTPase blocking TSC lysosomal mobility and rescuing TORC1 activity. Demonstrating further integration of the amino acid–sensing and growth factor–signaling pathways, we showed that the GATOR2 complex acts upstream of the Rag GTPase to promote both the activating phosphorylation of AKT and the AKT–dependent inhibitory phosphorylation of TSC2.
spnA, which retain meiotic DSBs into late stages of oogenesis, exhibit a profound reduction in TORC1 activity in the female germline (Figure 1), data that suggest that low TORC1 activity may be important for the efficient repair of meiotic DSB. In line with this hypothesis, we determined that GATOR1–mutant ovaries, which have high levels of TORC1 activity, exhibit many phenotypes consistent with the mis-regulation of meiotic DSBs, including an increase in the steady-state number of meiotic DSBs, the retention of meiotic DSBs into later stages of oogenesis, and the hyperactivation of p53, a transcription factor that mediates a highly conserved response to genotoxic stress (Figure 2). Importantly, RNAi depletions of Tsc1 phenocopied the GATOR1 ovarian defects. TSC1 is a component of the potent TORC1 inhibitor Tuberous Sclerosis Complex (TSC), confirming that the mis-regulation of meiotic DSBs observed in GATOR1–mutant oocytes is attributable to high TORC1 activity rather than to a TORC1–independent function of the GATOR1 complex. Further genetic analysis revealed that many of the phenotypes associated with high TORC1 activity observed in GATOR1–mutant ovaries are the result of the hyperactivation of the downstream TORC1 target S6K. We also demonstrated that GATOR1 impacts the repair, rather than the generation, of meiotic DSBs. Our data are particularly intriguing in light of similar meiotic defects observed in npr3 mutants in Saccharomyces cerevisiae. The results raise the possibility that GATOR1–mediated downregulation of TORC1 activity may be a common feature of the early meiotic cycle in many eukaryotes.

Genotoxic stress has been implicated in the deregulation of retrotransposon expression in several organisms, including Drosophila. In line with these studies, we found that, in GATOR1 mutants, the double-stranded breaks that initiate meiotic recombination trigger the deregulation of retrotransposon expression. Similarly,
it was previously shown that p53–mutant females de-repress retrotransposon expression during oogenesis, but, as observed in GATOR1 mutants, primarily in the presence of meiotic DSBs. Through epistasis analysis, we determined that p53 and GATOR1 act through independent pathways to repress retrotransposon expression in the female germline. Surprisingly, we found that depletions of the TORC1 inhibitor TSC in the female germline resulted in little or no increase in retrotransposon expression. The data raise the interesting possibility that GATOR1 regulates retrotransposon expression independently of TORC1 activity. Notably, GATOR1 components, but not TSC components, were recently identified in a high-throughput screen for genes that suppress LINE1 (Long Interspersed Element-1) expression in mammalian tissue-culture cells. Taken together, our data indicate that the GATOR1 complex opposes retrotransposon expression during meiosis in a pathway that functions in parallel to p53 in the female germline of Drosophila.

Publications

Collaborators
- Brian Calvi, PhD, *Indiana University, Bloomington, IN*
- R. Daniel Camerini-Otero, PhD, *Genetics and Biochemistry Branch, NIDDK, Bethesda, MD*
- Yikang Rong, PhD, *San Yat-sen University, Guangzho, China*
- Youheng Wei, PhD, *Yangzhou University, Yangzhou, Jiangsu, China*

Contact
For more information, email *mlilly@helix.nih.gov* or visit *https://irp.nih.gov/pi/mary-lilly.*
Neurosecretory Proteins in Neuroprotection and Neurodevelopment

Mechanism of sorting, transport, and regulated secretion of neuroproteins

The intracellular sorting of proneuropeptides and neurotrophins to the regulated secretory pathway (RSP) is essential for processing, storage, and release of active proteins and peptides in the neuroendocrine cell. We investigated the sorting of proopiomelanocortin (POMC, also known as pro-ACTH/endorphin), proinsulin, and brain-derived neurotrophic factor (BDNF) to the RSP. Our studies showed that these proproteins undergo homotypic oligomerization as they traverse the cell from the site of synthesis in the endoplasmic reticulum (ER) to the trans-Golgi network (TGN). In the TGN, the proproteins are sorted into the dense-core granules of the RSP for processing by prohormone convertases and carboxypeptidase E (CPE) and then secreted. We showed that the sorting of prohormones to the RSP occurs by a receptor-mediated mechanism. Site-directed mutagenesis studies identified a 3-D consensus sorting motif consisting of two acidic residues found in POMC, proinsulin, and BDNF. We identified the transmembrane form of CPE as an RSP sorting receptor that is specific for the sorting signal of these proproteins.

We also investigated the role of secretogranin III (SgIII) as a surrogate sorting receptor for membrane CPE in targeting POMC to the RSP. Using RNA interference (siRNA) to knock down SgIII or CPE expression in pituitary AtT20 cells, we demonstrated in both cases that POMC secretion via the constitutive secretory pathway was elevated. In double CPE–SgIII knock-down cells, elevated constitutive secretion of POMC and stimulated secretion of ACTH were perturbed. Thus, CPE mediates trafficking of POMC to the RSP; SgIII may play a compensatory role for CPE in POMC sorting to the RSP.

Transport of vesicles containing hormone or BDNF to the plasma membrane for activity-dependent secretion is critical for endocrine function and synaptic plasticity. We showed that the cytoplasmic tail of a transmembrane form of CPE in hormone- or BDNF–containing dense-core secretory vesicles plays an important role in their transport to the vesicles' release site. Overexpression of the CPE tail inhibited the movement of BDNF– and POMC/CPE–containing vesicles to the processes in hippocampal neurons and pituitary cells, respectively. The transmembrane CPE tails on the POMC/ACTION
BDNF vesicles interact with dynactin and the microtubule-based motors KIF1A/KIF3A to effect anterograde vesicle movement to the plasma membrane for activity-dependent secretion. Additionally, in collaboration with Joshua Park, we showed that another player, snapin, binds directly to the cytoplasmic tail of CPE and connects to the microtubule motor complex, consisting of kinesin-2 and kinesin 3 to mediate the post-Golgi anterograde transport of POMC/ACTH vesicles to the process terminals of AtT20 cells for secretion. Knockdown of snapin reduced stimulated ACTH secretion, while protein kinase A (PKA) activation by forskolin significantly increased the interactions of kinesin-2 and kinesin-3 with CPE and the levels of ACTH vesicles at the terminus and enhanced secretion of ACTH in AtT20 cells. Thus, our study has uncovered a novel complex consisting of the CPE cytoplasmic tail snapin-kinesin 2 and 3, which mediates anterograde transport of ACTH/POMC vesicles to the process terminals for secretion in a PKA-dependent manner in neuroendocrine cells.

Serpinin, a chromogranin A–derived peptide, regulates secretory granule biogenesis, cell survival, cardiac function, and angiogenesis.

Our previous studies in pituitary AtT-20 cells provided evidence that an autocrine mechanism up-regulates large dense-core vesicle (LDCV) biogenesis to replenish LDCVs following stimulated exocytosis of the vesicles. We identified the autocrine signal as serpinin, a novel 26 amino–acid, chromogranin A (CgA)–derived peptide cleaved from the C-terminus of CgA. Serpinin is released in an activity-dependent manner from LDCVs and activates adenyl cyclase to raise cAMP levels and protein kinase A in the cell. This leads to translocation of the transcription factor Sp1 from the cytoplasm into the nucleus and enhanced transcription of a protease inhibitor, protease nexin 1 (PN-1), which then inhibits granule protein degradation in the Golgi complex, stabilizing and raising granule protein levels in the Golgi and enhancing LDCV formation. We also identified modified forms of serpinin, pyroglutamyl-serpinin (pGlu-serpinin), and serpinin-RRG, a C-terminally extended form, in the secretion medium of AtT20 cells and in rat heart tissue. pGlu-serpinin is synthesized and stored in secretory granules and secreted in an activity-dependent manner from AtT20 cells. We observed pGlu-serpinin immunostaining in nerve terminals of neurites in mouse brain, olfactory bulb, and retina, suggesting a role as a neurotransmitter or neuromodulator. Additionally, pGlu-serpinin exhibited neuroprotective activity against oxidative stress in AT20 cells and against low K+-induced apoptosis in rat cortical neurons. In collaboration with Bruno Tota, we found that pGlu-serpinin has positive inotropic activity in cardiac function, with no change in blood pressure and heart rate. pGlu-serpinin acts through a β1-adrenergic receptor/adenylate cyclase/cAMP/PKA pathway in the heart. pGlu-serpinin and other CgA–derived cardio-active peptides thus emerge as novel β-adrenergic inotropic and lusitropic modulators. Together, they can play a key role in the myocardium’s orchestration of its complex response to sympatho-chromaffin stimulation. Additionally, pGlu serpinin is a powerful cardio-protectant after ischemia. The mechanism involves the activation of the reperfusion-injury salvage kinase (RISK) pathway. In collaboration with Angelo Corti, we showed that serpin-RRG had anti-angiogenic activity.

Role of CPE/NF–alpha1 in neuroprotection and anti-depression during stress

A null mutation in the CPE (also known as Neurotrophic factor-alpha1, NF-alpha1) gene in a woman and a homozygous non-sense mutation in three siblings of a different family were identified and found to display clinical features such as obesity, type 2 diabetes, learning disabilities, and hypogonadotrophic hypogonadism, indicating the importance of CPE in human disease. To study the physiological functions of CPE/NF-alpha1 in vivo, we generated a Cpe knock-out (KO) mouse. The KO mouse exhibited obesity, infertility, and diabetes. Further analysis of Cpe–KO mice revealed defects in learning and memory by the Morris water maze.

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and object-preference tests, and depressive-like behavior by the forced swim test. Electrophysiological measurements showed a defect in the generation of long-term potentiation in hippocampal slices. We discovered that a major cause of the neurological defects in these mice was the loss of CA3 neurons in the hippocampus after weaning stress. Hippocampal neurons in the CA3 region are enriched in CPE. Cpe-KO mice showed normal hippocampal cytoarchitecture at three weeks of age just before weaning, indicating that the defect was not a developmental problem. Rather, we hypothesized that the degeneration of the CA3 neurons was likely caused by glucocorticoid-induced epileptic-like neuronal firing of the granule cells in the dentate gyrus, releasing large amounts of glutamate during the weaning stress paradigm, which includes emotional stress from maternal separation, and physical stress from ear-tagging and tail clipping for genotyping. The hypothesis was supported by the finding that treatment with the carbamazepine, an anti-epileptic drug, prior to weaning prevented the stress-induced degeneration of the CA3 neurons in the Cpe-KO mice. Hence, CPE/NF-alpha1 is important for the survival of CA3 neurons during severe stress. To determine whether the neuroprotective effect of CPE/NF-alpha1 depends on the BDNF–TrkB pathway (Trk: tropomyosin receptor kinase), we treated mice with ANA12, a trkB inhibitor. Interestingly, downregulation of the BDNF–TrkB pathway had no detrimental effect on the survival of the CA3 neurons after the weaning stress paradigm, unlike the Cpe-KO mice, which showed complete degeneration, suggesting that CPE/NF-alpha1 is more critical than BDNF in protecting CA3 neurons from severe stress-induced cell death.

Furthermore, we showed that a mutant mouse expressing an enzymatically inactive form of CPE/NF-alpha1 (E342Q) had a normal hippocampus and learning and memory after the weaning stress paradigm, indicating that the neuroprotective action of CPE/NF-alpha1 is independent of its enzymatic activity [Reference 1]. We showed that CPE/NF-alpha1 (E342Q), either overexpressed or applied externally to cultured hippocampal or cortical neurons, protected the neurons from apoptosis induced by oxidative stress with hydrogen peroxide or glutamate treatment. Likewise, the enzymatically inactive form of CPE/NF-alpha1 (E342Q), applied extracellularly, had the same neuroprotective effect. We thus demonstrated that CPE/NF-alpha1 acts extracellularly as a signaling molecule to mediate neuroprotection. To this end, we showed that ¹²⁵I-CPE/NF-alpha1 bound specifically to the cell surface of HT22 cells, an immortalized hippocampal neuronal cell line, in a saturable manner, with a Kd 4.37nM, suggesting the existence of a receptor. K235a, a Trk family inhibitor, and PD16285, a fibroblast growth factor receptor (FGFR1-3) inhibitor, did not prevent the neuroprotective action of CPE/NF-alpha1 in hippocampal neurons treated with H₂O₂, suggesting that CPE/NF-alpha1 likely uses a different class of receptors than those of the Trk family or FGFRs. Work is now in progress to identify the receptor.

The mechanism of action of CPE/NF-alpha1 in neuroprotection involves the activation of the ERK1/2 (extracellular-signal-regulated kinase) signaling pathway and the Akt signaling pathway (an intracellular signal transduction pathway) during stress, which then leads to enhance CPE/NF-alpha1 expression of a pro-survival mitochondrial protein, Bcl2, inhibition of caspase 3 activation, and promotion of neuronal survival [Reference 1]. Furthermore, this CPE/NF-alpha1–mediated neuroprotection pathway is activated by rosiglitazone, a PPARγ ligand (a peroxisome proliferator-activated receptor, a transcription factor), which binds to PPARγ binding sites in the CPE promoter. Examination of the pathway during stress in vivo revealed that, after mild chronic restraint stress (CRS) for 1 hour per day for seven days, mice showed significantly elevated levels of CPE/NF-alpha1 mRNA and protein, as well as the anti-apoptotic protein Bcl2, in the hippocampus. In situ hybridization studies indicated especially elevated CPE/NF-alpha1 mRNA levels in the CA3 region and no gross neuronal cell death after mild CRS. Furthermore, primary hippocampal neurons in culture showed
elevated CPE/NF-alpha1 and Bcl2 expression and a decline in Bax, a proapoptotic protein, after treatment with the synthetic glucocorticoid dexamethasone. The up-regulation was mediated by glucocorticoid binding to glucocorticoid-regulatory element (GRE) sites on the promoter of the Cpe gene. Thus, during mild CRS, when glucocorticoid is released, CPE/NF-alpha1 and Bcl2 expression are coordinately up-regulated to mediate neuroprotection of hippocampal neurons. The importance of CPE as a neuroprotective agent was demonstrated by the absence of an increase in Bcl2 in the hippocampus of Cpe–KO mice after CRS, leading to the degeneration of the CA3 neurons. Furthermore, CRS also elevated the expression of the signaling protein FGF2. We demonstrated that primary hippocampal neurons treated with CPE/NF-alpha1 increased FGF2 expression. Thus, another pathway for CPE/NF-alpha1 action may be through FGF2, which is known to have neuroprotective effect. In summary, CPE/NF-alpha1 is a critical neurotropin for protecting CA3 neurons against stress-induced cell death via Erk–BCL2 pathway.

The relevance of CPE/NF-alpha1 in neuroprotection in humans was underscored by our studies on a mutation of the CPE gene found in an Alzheimer’s disease (AD) patient [Reference 2]. Our search in the GenBank EST database identified a sequence entry from the cortex of an AD patient that had three adenosine inserts in the CPE gene, thereby introducing nine amino acids, including two glutamines, into the mutant protein, herein called CPE–QQ. Expression of CPE–QQ in Neuro2a cells indicates that it is not secreted. Co-expression of wild-type (WT) CPE and CPE–QQ in Neuro2a cells resulted in degradation of both forms of the protein and reduced secretion of WT CPE. Immunocytochemical studies show that CPE–QQ accumulated in the perinuclear region of the cells and co-localized with Calnexin, an ER marker. Moreover, cells expressing CPE–QQ were unhealthy and appeared to be undergoing ER stress, unlike the cells expressing WT CPE. CPE–QQ was not secreted and even prevented WT CPE from being secreted by aggregating with it. Overexpression of CPE–QQ in rat primary hippocampal neurons resulted in elevated levels of the ER–stress marker CHOP, reduced levels of the pro-survival protein Bcl-2, and increased neuronal cell death. Thus, CPE–QQ induces cell death through ER stress and down-regulation of Bcl-2 expression. We then generated transgenic mice overexpressing CPE–QQ and showed that, at 50 weeks but not at 11 weeks of age, the animals exhibited memory deficits and depressive-like behavior compared with WT mice, but that their spatial learning ability was unimpaired. The CPE–QQ mice were neither obese nor diabetic, likely owing to some CPE activity in these mice, given that the endogenous WT gene was not deleted. However, they had significantly fewer neurites in the CA3 region, the dentate gyrus of the hippocampus, and in the medial prefrontal cortex, indicative of neurodegeneration. Moreover, they exhibited reduced neurogenesis in the subgranular zone and hyperphosphorylation of the microtubule-associated protein tau at ser395, a hallmark of AD. The studies identified a human mutation in the CPE gene resulting in expression of a CPE–QQ protein, which caused neurodegeneration and impairment of memory function, as well as depressive-like behavior in a mouse model, linking this gene for the first time to neurodegenerative disease and depression [Reference 2].

Stress also induces depression. Huda Akil’s group (University of Michigan) reported that FGF2 is an anti-depressant. We found that prolonged (6 hours per day for 21 days) restraint stress reduced CPE/NF-alpha1 and FGF2 in the hippocampus of mice and induced depressive-like behavior. However, after short-term restraint stress (1 hour per day for 7 days), mice did not show depressive-like behavior despite elevated corticosterone levels indicative of stress. Moreover, hippocampal CPE/NF-alpha1, FGF2, and doublecortin, a marker for neurogenesis, were elevated in these mice, suggesting that the anti-depressive effects of CPE/NF-alpha1 are mediated, at least in part, through increased neurogenesis. Indeed, we found that exogenously applied CPE/NF-alpha1 could up-regulate FGF2 mRNA and protein expression in cultured hippocampal neurons,
indicating that CPE/NF-alpha1 regulates FGF2 expression. CPE/NF-alpha1–KO mice exhibited severely reduced hippocampal FGF2 levels and immature neuron numbers in the subgranular zone. The mice displayed depressive-like behavior, which was rescued by FGF2 administration. Thus, we propose that CPE/NF-alpha1 prevents stress-induced depressive-like behavior by up-regulating hippocampal FGF2 expression, which leads to enhanced neurogenesis and anti-depressive activity [Reference 3]. Furthermore, we found that rosiglitazone, an anti-diabetic drug, can trigger this pathway [Reference 3]. Rosiglitazone has previously been shown to be effective in treating diabetic patients with bi-polar disorders.

**Role of CPE/NF-alpha1 and CPE-deltaN in embryonic brain development**

Embryonic mouse brains express three forms of CPE/NF-alpha1 mRNA (2.1kb, 1.9kb, and 1.73kb in size), which encode a 53kD wild-type CPE/NF-alpha1, and two terminal-truncated isoforms of CPE/NF-alpha1-DN (47kD and 40kD). The three mRNAs are expressed as early as E8.5 and increase significantly in two waves at E10.5 and postnatal day 1 [Reference 4]. Interestingly, CPE/NF-alpha1-DNs are not expressed in adult mouse brain. *In situ* hybridization studies indicate that CPE/NF-alpha1 is expressed primarily in the forebrain in mouse embryos, suggesting that CPE/NF-alpha1 plays a role in neurodevelopment. We examined the effect of CPE/NF-alpha1 on E13.5 neocortex-derived neurospheres, which contain stem cells and neuroprogenitors. Application of recombinant CPE/NF-alpha1 reduced the number and size of the neurospheres formed, suggesting inhibition of proliferation and maintenance of the ‘stemness’ of the stem cells in the neurospheres. CPE/NF-alpha1 down-regulated the wnt pathway in the neurospheres, leading to reduced levels of beta-catenin, a protein known to enhance proliferation, suggesting that CPE/NF-alpha1’s inhibitory effect on proliferation is brought about by negatively regulating the wnt pathway.

We also carried out differentiation studies using neurospheres from seven-day cultures that were dissociated into single cells and cultured for an additional five days. We observed an increase in astrocytes after CPE/NF-alpha1 treatment, without alteration in the percentage of neuronal and oligodendrocyte populations. We also observed the phenomenon when the cultured embryonic stem cells were treated with a non-enzymatic form of CPE, indicating that the effect was independent of enzymatic activity. Interestingly, dissociated cells from neurospheres derived from Cpe/NFalpha1–KO mouse embryos showed fewer astrocytes but more neurons, which was reversed with CPE/NF-alpha1 application. *In vivo*, Cpe/NF-alpha1–KO mouse cortex (at P1, the time of astrocytogenesis) showed about half the astrocyte numbers of those of WT animals, confirming the *ex vivo* data. Our results suggest a novel role for CPE/NF-alpha1 as an extracellular signal to inhibit proliferation and induce differentiation of neural stem cells into astrocytes, thus playing an important role in neurodevelopment [Reference 5].

Neurite outgrowth is key to the formation of synapses and the neural network during development. We found that CPE/NF-alpha1 prevented Wnt-3a inhibition of nerve growth factor (NGF)–stimulated neurite outgrowth in PC12 cells, a neuroendocrine cell line, and in cortical neurons. Moreover, CPE/NF-alpha1 augmented Wnt-5a–mediated neurite outgrowth. Thus, the interplay between NGF preventing neurite outgrowth, which is inhibited by Wnt-3a, and augmenting neurite outgrowth, which is mediated by Wnt-5a and CPE/NF-alpha1, could play an important role in regulating these positive and negative cues, which are critical for neurodevelopment. Analysis of the brain of 6- to 14-week-old Cpe–KO mice revealed poor dendritic pruning in cortical and hippocampal neurons, which could affect synaptogenesis.
We also studied the function of 40kD CPE/NF-alpha1-DN and showed that it is translocated from the cytoplasm into the nucleus of rat embryonic neurons. Overexpression of 40kD CPE/NFalpha1-DN in HT-22 cells, a hippocampal cell line, resulted in an increase in expression of IGF binding protein2 (IGFBP2), Death-Associated Protein (DAP1), and Ephrin 1A mRNAs, proteins and an mRNA that are involved in neuronal proliferation, programmed cell death, and neuronal migration, respectively. We demonstrated that IGFBP2 is involved in proliferation in a CPE/NF-alpha1-DN–dependent manner in HT22 and mouse cortical neurons [Reference 4]. Thus 40kD CPE/NF-alpha1-DN functions to regulate expression of genes important in neurodevelopment. Further studies aimed at determining the role of CPE/NF-alpha1-DN in vivo are in progress.

**Publications**


**Collaborators**

- Angelo Corti, MD, *San Raffaele Scientific Institute, Milan, Italy*
- Beata Lecka-Czernik, PhD, *University of Toledo, Toledo, OH*
- Joshua J. Park, PhD, *University of Toledo, Toledo, OH*
- Bruno Tota, MD, *Università della Calabria, Cosenza, Italy*

**Contact**

For more information, email *lohp@mail.nih.gov* or visit *http://scn.nichd.nih.gov*. 
The Molecular Mechanics of Eukaryotic Translation Initiation

The goal of our research is to elucidate the molecular mechanisms underlying the initiation phase of protein synthesis in eukaryotic organisms. We use the yeast *Saccharomyces cerevisiae* as a model system and employ a range of approaches, from genetics to biochemistry to structural biology, in collaboration with Alan Hinnebusch's and Tom Dever's labs and several other research groups around the world.

Eukaryotic translation initiation is a key control point in the regulation of gene expression. It begins when an initiator methionyl tRNA (Met-tRNAi) is loaded onto the small (40S) ribosomal subunit. Met-tRNAi binds to the 40S subunit as a ternary complex (TC) with the GTP–bound form of the initiation factor eIF2. Three other factors, eIF1, eIF1A, and eIF3, also bind to the 40S subunit and promote the loading of the TC. The resulting 43S preinitiation complex (PIC) is then loaded onto the 5’-end of an mRNA with the aid of eIF3 and the eIF4 group of factors: the RNA helicase eIF4A; the 5’-7-methylguanosine cap-binding protein eIF4E; the scaffolding protein eIF4G; and the 40S subunit– and RNA–binding protein eIF4B. Both eIF4A and eIF4E bind to eIF4G and form the eIF4F complex. Once loaded onto the mRNA, the 43S PIC is thought to scan the mRNA in search of an AUG start codon. The process is ATP–dependent and likely requires several RNA helicases, including the DEAD–box protein Ded1p. Recognition of the start site begins with base pairing between the anticodon of tRNAi and the AUG codon. Base pairing then triggers downstream events that commit the PIC to continuing initiation from that point on the mRNA, events that include ejection of eIF1 from its binding site on the 40S subunit, movement of the C-terminal tail (CTT) of eIF1A, and release of phosphate from eIF2, which converts eIF2 to its GDP–bound state. In addition, the initiator tRNA moves from a position that is not fully engaged in the ribosomal P site [termed P(OUT)] to one that is [P(IN)], and the PIC as a whole converts from an open conformation that is conducive to scanning to a closed one that is not. At this stage, eIF2•GDP dissociates from the PIC, and eIF1A and a second GTPase factor, eIF5B, coordinate joining of the large ribosomal subunit to form the 80S initiation complex. In a process that appears to result in conformational reorganization of the complex, eIF5B hydrolyzes GTP and then dissociates along with eIF1A.
The molecular mechanics of eukaryotic translation initiation

In 2020, we made significant progress on our two main projects, despite significant slowdowns owing to the COVID-19 pandemic. We further developed the “Rec-Seq” methodology, which allows transcriptome-wide measurement of translational efficiencies in a fully reconstituted yeast translation initiation system. Unlike the in vivo approach of ribosome profiling, Rec-Seq allows us to isolate events that are directly part of translation initiation and separate them from other connected processes such as transcription, mRNA export from the nucleus, and mRNA decay. In addition, Rec-Seq has an internal control that allows us to make absolute measurements of translation initiation efficiencies rather than only being able to make relative assessments. We can also easily omit factors, change their concentrations, and substitute mutant versions of components, including those that are lethal in vivo, and assess the effects of the changes.

We used the method to probe the functions of several factors that play roles in recruitment of mRNAs to the ribosomal PIC. Our data indicate that the DEAD-box RNA helicase eIF4A is required for efficient recruitment of nearly all cellular mRNAs, whereas Ded1, another DEAD-box RNA helicase, only promotes recruitment of a subset of mRNAs. In particular, mRNAs with long, structured 5′-untranslated regions (5′-UTRs) depend on Ded1 for recruitment to the PIC, whereas mRNAs with short, unstructured 5′-UTRs do not, data that support the idea that Ded1 plays a role in unwinding secondary structures in 5′-UTRs, while eIF4A has a more general role in promoting mRNA loading onto the PIC. We also showed that the factors eIF4B and eIF4G-eIF4E play general stimulatory roles in mRNA recruitment rather than having mRNA specificity.

More recently, we expanded the Rec-Seq approach to allow us to measure the kinetics of mRNA recruitment by analyzing several timepoints (Figure 1), an advance that provides us with unprecedented resolution in terms of understanding the influences of different factors and RNA features on the process of mRNA recruitment to the PIC. Using the approach, our initial data demonstrated that the mRNAs that are recruited the fastest to the PIC are those with short, unstructured 5′-UTRs, whereas those that are recruited the slowest have long, structured 5′-UTRs. The data are consistent with the idea that the unwinding of structures in the 5′-UTR is a
We also made significant progress in our work to elucidate the functions of the unstructured N-terminal tails (NTTs) of the translation initiation factors eIF1 and eIF5. In particular, we completed the initial phenotypic analyses of alanine scanning mutagenesis through the NTT of eIF1. The results reveal many positions at which substitution of the wild-type residue with alanine produces strong phenotypes related to recognition of the mRNA start codon (Figure 2), indicating that the eIF1 NTT plays an important role in start-codon recognition. We expressed and purified key NTT mutants and have begun to study the effects of the mutations on steps in the initiation process in vitro, using the fully reconstituted system. We expect that these studies will shed a great deal of light on the mechanism of action of the eIF1 NTT in start-codon recognition.

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**Publications**
Collaborators

• Thomas Dever, PhD, Section on Protein Biosynthesis, NICHD, Bethesda, MD
• Alan Hinnebusch, PhD, Section on Nutrient Control of Gene Expression, NICHD, Bethesda, MD
• Venkatraman Ramakrishnan, PhD, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Contact

For more information, email jon.lorsch@nih.gov or visit https://irp.nih.gov/pi/jon-lorsch.
Our research focuses on the development of the mammalian hematopoietic system. A long-term area of interest for our laboratory is the study of signal transduction molecules and pathways that regulate T lymphocyte maturation in the thymus. Currently, we are analyzing transgenic and conditional non-signaling mutants of T cell antigen receptor (TCR) signaling subunits, made in our lab, to evaluate the importance of individual TCR signaling chains and motifs at specific stages of T cell development and in mature T cells. We are also investigating whether modification of TCR signaling subunits can be used to enhance the tumoricidal activity of T cells. Using gene profiling, we are seeking to identify proteins that are important for fine-tuning the T cell–signaling response in developing and mature T lymphocytes. In conjunction with checkpoint inhibitors for immunotherapy in humans, such molecules may also be targets to enhance anti-tumor activity. We are also investigating the function of new T cell–specific proteins that we identified by subtraction library screening. Our studies revealed a critical role for one of these proteins, called Themis, in T cell development by enhancing the TCR–signaling response in thymocytes. We found that another newly identified protein, Fbxl12, is important for regulating proliferation during early T cell development. Another area of investigation focuses on hematopoietic stem cells (HSCs) and early stages of T cell, B cell, and erythrocyte development. We initiated characterization of a protein (Ldb1) that is important for the generation and maintenance/self-renewal of HSCs, which revealed a critical function for Ldb1 as a key subunit of multimeric DNA–binding complexes in controlling the self-renewal/differentiation cell-fate decision in HSCs. Current work, which involves genome-wide screening by RNA-seq and ChIP-seq, seeks to extend our knowledge of the role of Ldb1 complexes in regulating gene transcription and to explore the function of such complexes in other hematopoietic lineages. For example, we have begun to investigate the importance of Ldb1 complexes for regulating self-renewal in immature thymocytes and in the induction of T cell acute lymphoblastic leukemia (T-ALL). Our results suggest that Ldb1 complexes represent potential therapeutic targets for the treatment of an aggressive form of childhood leukemia called early T progenitor T cell acute lymphoblastic leukemia (ETP-T-ALL).
T cell antigen receptor signaling in thymocyte development

Much of our research has focused on the role of TCR signal transduction in thymocyte development. Signal transduction sequences, termed immunoreceptor tyrosine-based activation motifs (ITAMs), are contained within four distinct subunits of the multimeric TCR complex (CD3-zeta, CD3-gamma, CD3-delta, and CD3-epsilon). Di-tyrosine residues within ITAMs are phosphorylated upon TCR engagement; their function is to recruit signaling molecules, such as protein tyrosine kinases, to the TCR complex, thereby initiating the T cell–activation cascade. Though conserved, ITAM sequences are nonidentical, raising the possibility that the diverse developmental and functional responses controlled by the TCR may be partly regulated by distinct ITAMs through the recruitment of different effector molecules. We previously generated CD3-zeta–deficient and CD3-epsilon–deficient mice by gene targeting. We genetically reconstituted the mice with transgenes encoding wild-type or signaling-deficient (ITAM–mutant) forms of CD3-zeta and CD3-epsilon and characterized the developmental and functional consequences of the alterations for TCR signaling. We found that TCR–ITAMs are functionally equivalent but act in concert to amplify TCR signals and that TCR signal amplification is critical for thymocyte selection, the process by which potentially useful immature T cells are instructed to survive and differentiate further (positive selection) and by which potentially autoreactive cells, which may cause autoimmune disease, are deleted in the thymus (negative selection).

We also found that a complete complement of TCR–ITAMs is not required for most mature T cell effector functions. However, recent work demonstrated a requirement for ITAM multiplicity for the generation of T follicular helper cells, which are necessary for optimal B cell antibody responses. One possible explanation for the relatively mild phenotype observed in the TCR ITAM–reduced mice is that ITAM–mediated signal amplification is not required for most mature T cell activation responses; another is that, in ITAM–mutant mice, T cells exhibit normal functional responsiveness because of compensatory mechanisms (such as regulated expression of other signaling molecules) imposed during development. To resolve this question, we recently generated a TCR–zeta chain conditional knock-in mouse in which T cell development and selection can occur.
without attenuation of TCR signaling (i.e., in the presence of a wild-type 3-ITAM “6Y” zeta chain), but in which mature, post-selection T cells may be induced to express TCRs containing signaling-defective (0-ITAM “6F”) zeta chains in lieu of wild-type zeta chains (Figure 1). Thus, mature T cell signaling should not be influenced by potential compensatory mechanisms that operate during T cell maturation, and T cells in such mice should be faithful indicators of the role of multiple TCR ITAMs in mediating specific, mature T cell responses. Preliminary experiments confirmed that the knock-in zeta locus functions as predicted. We are currently evaluating the effect of late ‘switching’ from 6Y zeta to 6F zeta in mature T cells generated with wild-type 6Y zeta containing TCRs. The experimental conditions will more closely mimic those in which TCR signaling is attenuated pharmacologically to treat human diseases such as autoimmunity. In addition, the experiments should provide information relevant to the design of engineered tumor antigen–specific TCRs and chimeric antigen receptor T cells (CAR T cells), which currently are configured to express zeta ITAM signaling module(s).

Identification and characterization of proteins important for TCR fine tuning and TCR signaling

We extended our analysis of TCR–signaling subunits to other molecules that participate in or influence the TCR–signaling response. The cell-surface protein CD5 negatively regulates TCR signaling and functions in thymocyte selection. Examination of CD5 expression during T cell development revealed that surface levels of CD5 are regulated by TCR signal intensity and by the affinity of the TCR for self-peptide ligands in the thymus that mediate selection. To determine whether the ability to regulate CD5 expression is important for thymocyte selection, we generated transgenic mice that constitutively express high levels of CD5 throughout development. Over-expression of CD5 significantly impaired positive selection of some thymocytes (those that would normally express low levels of CD5) but not of others (those that would normally express high levels of CD5). The findings support a role for CD5 in modulating TCR signal transduction and thereby influencing the outcome of thymocyte selection. Current studies center on identifying the mechanism by which CD5 inhibits TCR signaling and on determining whether the protein's regulated expression during development is important for preventing autoimmunity. For that purpose, we generated a conditional CD5 deletion mouse in which CD5 expression can be removed before, during, or after T cell development. The ability of individual thymocytes to regulate CD5 expression represents a mechanism for ‘fine tuning’ the TCR–signaling response during development so that the integrated signaling response can be adjusted to permit T cell functional competency without causing autoimmunity. Reasoning that, in addition to CD5, other molecules participate in TCR tuning, we initiated microarray-based screening for genes differentially expressed in developing T cells under conditions of high- or low-affinity TCR interactions. We identified several genes from this screen for further study and are validating their function as tuning molecules. Given that the molecules regulate TCR signaling, they represent potential autoimmune-disease susceptibility markers and potential targets for treatment of patients with cancer or autoimmune disease, similar to current ‘checkpoint inhibitor’ therapies that are based on blocking the function of the induced inhibitory molecules PD-1 and CTLA-4.

Identification and characterization of Themis, a novel protein required for T cell development

Using a subtractive cDNA library–screening approach, we identified Themis, now known as Themis1, a novel T cell–specific adapter protein (Figure 2). To investigate the function of Themis1 in T cell signaling and development, we generated Themis1 knock-down cell lines, Themis1 knock-out mice (conventional and conditional), and Themis1 transgenic mice. Analysis of the effects of modulating Themis1 expression revealed a critical role for the protein in late T cell development. We obtained the following results. First, the Themis1...
paralog Themis2, which is expressed in B cells, can substitute for Themis1 in T cell development. We found that the ability of the B cell–specific family Themis member Themis2 was equivalent to that of Themis1 to restore normal T cell development in Themis1–/– mice, thus demonstrating functional redundancy of Themis1 and Themis2. Second, we generated retroviruses encoding domain-deletion mutants of Themis1, infected Themis1–/– bone marrow progenitors, and made bone marrow chimeras to determine which regions of Themis1 are important for in vivo function. We found that the Themis1 proline-rich sequence (PRS), which mediates binding to the signaling protein Grb2, was required for in vivo function, as assessed by rescue of the developmental block in Themis1–/– thymocytes, but that the CABIT (cysteine-containing, all beta in Themis)–domain cysteines are not essential. Third, we generated Themis2–/– mice and began a collaboration with Richard Cornall to characterize the mice. Our results identified an important role for Themis2 in facilitating B cell activation by low-avidity, but not high-avidity, B cell receptor (BCR)–antigen interactions. Themis2 was required to elicit normal Ca2+ signaling via the Erk pathway in response to low-avidity interactions and was necessary for positive selection of B1 cells and germinal-center B cells by self and foreign antigens. We detected Themis2 in complexes with the signaling proteins Grb2, Lyn, and PLCgamma2 and found that Themis2 is required for normal tyrosine phosphorylation of Lyn and PLCgamma2. This subtle but clear phenotype of Themis2–/– mice was not detected in a previous and less extensive study of Themis2–/–, which concluded that loss of Themis2 has no effect on B cell development or function.

Our findings show that the impact of loss of Themis1 and Themis2 on T and B cell development, respectively, is strikingly similar. In each case, the main effect is on positive selection, which is controlled by low-avidity antigen-receptor interactions. This, together with the ability of Themis2 to rescue T cell development in Themis1–/– mice, indicates that Themis1 and Themis2 perform similar functions in T and B cells.

In the past few years, we focused on determining the molecular function of Themis1. Themis1, Themis2, and a large family of related metazoan proteins contain a novel globular domain of unknown function called the CABIT module (see above and Figure 2). Using cell transfection, biochemical, and protein assay techniques, we determined that CABIT modules bind to the catalytic domain of SHP-1, a key hematopoietic protein tyrosine phosphatase. In the presence of reactive oxygen species (ROS), which are generated in activated T cells, Themis1, via its CABIT module, promoted oxidation of the SHP-1 catalytic cysteine and therefore

**FIGURE 2.** Themis is highly conserved in vertebrates.

Themis contains two novel CABIT domains, each with a conserved cysteine (red) and conserved flanking residues (yellow), a nuclear localization signal (NLS), and a proline-rich region (PRR).
inactivated SHP-1. The CABIT modules from all five mammalian Themis-family proteins also inhibited SHP-1, indicating that this activity was common to the CABIT module. Given that SHP-1 is an inhibitory phosphatase that functions to dampen TCR signaling by de-phosphorylating several targets, including protein tyrosine kinases, the finding established an activating function for Themis1 in cell signaling through its ability to bind to and inhibit SHP-1. Interestingly, Themis1 is highly expressed in developing thymocytes at the stage at which they undergo positive selection. It had been known for years that thymocytes are more sensitive to TCR stimulation than are mature T cells, but the reason for this sensitivity was unknown. The function of Themis1, together with its high expression in thymocytes, provides an explanation for the sensitivity of thymocytes to TCR signaling. By showing that deletion of the gene encoding SHP-1 rescues T cell development in Themis1\(-/\)-mice, we confirmed that the primary role of Themis1 is to inhibit SHP-1. In addition to identifying the function of CABIT modules, our results provide insight into the role of other metazoan CABIT–containing proteins (which number in the hundreds). Our ongoing studies are focusing on further characterization of CABIT proteins and determining their role in development and their possible involvement in human disease.

Role of the F-box protein Fbxl12 in thymocyte development

A major aspect of the T cell maturation process is the precise regulation of cell proliferation. Rather than being a shared property among all or most developing thymocytes, proliferation is strictly limited to two stages during early development. The initial proliferative phase is driven by thymus-expressed cytokines, the second coincides with ‘beta selection’ (i.e., is initiated in cells that have productively rearranged the TCR beta chain and express a signaling complex called the pre-TCR). The proliferative burst that accompanies beta selection is estimated to result in a 100–200 fold expansion and is essential for further differentiation and for maximizing TCR diversity. Previous work showed that beta selection–associated proliferation requires concurrent signals by the pre-TCR and the Notch receptor, but how these signals induce cell-cycle progression and why they need to be coordinated has remained unclear. Initiation of proliferation in beta-selected thymocytes requires the ubiquitin-mediated degradation of the cyclin-dependent kinase inhibitor Cdkn1b, which acts to prevent cell-cycle progression. In a recent study, we examined the molecular control of beta selection–associated proliferation. We confirmed prior findings that Cdkn1b degradation is induced by an SCF E3 ubiquitin ligase that contains the ligand-recognition subunit Fbxl1. Deletion of Fbxl1 partially blocked beta selection–associated proliferation, a defect that was rescued by co-deletion of Cdkn1b. We identified a new F-box protein, Fbxl12, that is highly expressed in thymocytes. We found that Fbxl12 also functions as an SCF E3 ligase subunit, which, like Fbxl1, directs Cdkn1b degradation. The phenotype of Fbxl12–deficient mice generated in our lab was strikingly similar to Fbxl1–deficient mice, and deletion of both Fbxl1 and Fbxl12 resulted in a severe block in beta selection–associated proliferation, indicating that Fbxl1 and Fbxl12 act in concert to regulate thymocyte proliferation. Interestingly, we found that Fbxl1 expression is induced by Notch signaling, whereas Fbxl12 expression is induced by pre-TCR signaling. Both Fbxl1 and Fbxl12 are required for thymocyte proliferation; thus, their selective regulation by Notch and the pre-TCR, respectively, provides an explanation for why concurrent Notch and pre-TCR signaling are necessary for cell-cycle progression and proliferation at the beta selection checkpoint.

Role of Ldb1 transcription complexes in hematopoiesis and in T cell acute lymphoblastic leukemia

Lim domain binding protein-1 (Ldb1) is a ubiquitously expressed nuclear protein that contains a LIM–zinc finger protein–interaction motif and a dimerization domain. In hematopoietic cells, Ldb1 functions by interacting with and/or recruiting specific partners (including the LIM–only protein Lmo2 and the transcription factors
Ldb1 forms a multimeric DNA–binding complex in hematopoietic cells with the adapter Lmo2 and the transcription factors Scl and Gata1 or Gata2. In hematopoietic stem cells (HSCs), in which Gata2 is highly expressed, Ldb1-Lmo2-Scl-Gata2 complexes positively regulate expression of HSC maintenance genes. Differentiation of HSCs to the myeloid or lymphoid lineage (LMPP) is triggered by downregulation of Ldb1 complexes, whereas commitment to the erythroid lineage (ery) is triggered by induction of Gata1 and downregulation of Gata2, resulting in the formation of an Ldb1-Lmo2-Scl-Gata1 complex, which positively regulates expression of erythroid-specific genes.

Lyl1 or Tal1 and Gata1 or Gata2) to form multi-molecular transcription complexes (Figure 3). Within the hematopoietic lineage, expression of Ldb1 is highest in progenitor cells, which include hematopoietic stem cells (HSCs). Ldb1–null (Ldb1–/–) mice die between day 9 and 10 of gestation, preventing us from directly studying the impact of loss of Ldb1 on fetal or adult hematopoiesis. We investigated the role of Ldb1 in hematopoiesis by following the fate of Ldb1–/– embryonic stem cells (ESCs) in mouse blastocyst chimeras and by conditional, stage-specific deletion of Ldb1. Significantly, Ldb1–/– ESCs were capable of generating HSCs, which could give rise to both myeloid and lymphoid lineage cells; however, the number of Ldb1–/– HSCs gradually diminished at later stages of development. Following adoptive transfer of fetal liver hematopoietic progenitor cells, Ldb1–/– HSCs were rapidly lost, indicating a failure of self renewal or survival. More recent data indicate that the loss of Ldb1–/– HSCs results from differentiation rather than cell death. Although expressed in ESCs, Ldb1 is not required for ESC maintenance, indicating a selective requirement in adult stem-cell populations. We performed a genome-wide screen for Ldb1–binding sites using ChIP-seq. Analysis of the ChIP-seq data revealed that Ldb1 complexes bind at the promoter or at regulatory sequences near a large number of genes known to be required for HSC maintenance. The data suggest that Ldb1 complexes function in a manner similar to Oct4/nanog/Sox2, transcription factors that are all essential in maintaining the pluripotent ESC phenotype, to regulate a core transcriptional network required for adult stem cell maintenance. Examination of the function of Ldb1 in cell lineages downstream of the HSC identified an essential function in the erythroid lineage but not in myeloid cells or lymphoid cells. Interestingly, ChIP-seq analysis of Ldb1 DNA–binding complexes demonstrated that, in HSCs, Ldb1 complexes contain the transcription factor Gata2, whereas, in erythroid progenitors, Ldb1 complexes contain Gata1 (which is highly expressed in the erythroid lineage). The results indicate that multimeric Ldb1 transcription complexes have distinct functions in the hematopoietic system depending on their subunit composition, with Gata2–containing complexes regulating expression of HSC–maintenance genes and Gata1 complexes regulating expression of erythroid-specific genes (Figure 3). Current studies aim to determine how Ldb1 complexes regulate gene expression and the role of Ldb1 dimerization in mediating long-range promoter-enhancer interactions in hematopoietic cells. In addition, we are investigating a potential role for Ldb1 in regulating self renewal of T cell progenitors in the thymus.
Acute lymphoblastic leukemias are the most common type of cancer in children. T cell acute lymphoblastic leukemia (T-ALL) results from oncogenic transformation of immature T cell progenitors (thymocytes). Mouse models of T-ALL have been generated, and one of the most informative is the Lmo2-transgenic (Lmo2-tg) mouse, which expresses high levels of the nuclear adapter Lmo2 in thymocytes. The model closely mimics a prevalent type of human T-ALL, which is associated with chromosomal mutations that result in increased expression of LMO2. We recently reported that overexpression of Lmo2 in mouse thymocytes induces T-ALL at two distinct stages of development (an early ‘ETP’ stage and a later ‘DN3’ stage). Notably, human T-ALLs can also occur at two similar stages of thymocyte maturation. The most immature forms of T-ALL in Lmo2-tg mice and in humans express high levels of the transcription factor Hhex and are designated early T cell progenitor (ETP) T-ALL, whereas later-stage tumors are low in Hhex but express high levels of more mature markers of T cell development, including Notch1, Dtx1, Ptcra, and Hes1. Lmo2 functions as a subunit of the multimeric Ldb1–nucleated DNA–binding complexes described above. We found that normal ETP thymocyte progenitor cells express the same Ldb1 complex subunits that are present in HSCs and that ETPs exhibit HSC characteristics, including the self-renewal potential. ETPs in Lmo2-tg mice appear to be ‘locked’ into a pattern of perpetual self renewal and are refractory to normal inductive signals that promote further differentiation. Hhex is a target of Ldb1 complexes in HSCs and ETPs, a result that strongly suggests that Ldb1 complexes are responsible for the aberrant self renewal in Lmo2-tg mice that predisposes to oncogenesis. We hypothesized that Ldb1 complexes regulate self renewal in ETPs as well as in HSCs. Lmo2 is normally down-regulated when thymocytes undergo T lineage commitment, suggesting that extinguishing expression of Lmo2 (and by extension, Ldb1 complexes) is important for T cell differentiation and that failure to do so predisposes to oncogenesis via ‘second-hit’ transforming events.

In RNA-seq gene expression experiments, we found that the RNA expression signatures of Lmo2-tg immature thymocytes and HSCs are very similar, consistent with the notion that Lmo2 overexpression ‘freezes’ cells in a stem-cell self-renewal state. To determine whether Ldb1 complexes are in fact required for ETP self renewal and to explore the genes regulated by these complexes, we conditionally deleted Ldb1 in Lmo2-tg mice. We found that Ldb1 is required for Lmo2-tg–induced thymocyte self renewal and T-ALL induction, indicating that Lmo2 overexpression promotes T-ALL by functioning as a subunit of Ldb1 complexes. Currently, we are addressing several key questions, including whether, as predicted, Ldb1, and by extension Ldb1 complexes, regulate expression of genes that control a self-renewal genetic program in ETPs and whether Ldb1 complexes are necessary for the transcriptional/developmental effects of Lmo2. We are also determining the subunit structure and binding sites of Ldb1 complexes expressed in Lmo2-tg thymocytes. We anticipate that our results will provide insights into the mechanisms controlling T-ALL oncogenesis in humans and may thus provide new therapeutic avenues for the treatment of this devastating pediatric disease.

**Additional Funding**
- NICHD Director’s Award

**Publications**


**Collaborators**

- Cheryl Arrowsmith, PhD, *University of Toronto, Toronto, Canada*
- Remy Bosselut, PhD, *Laboratory of Immune Cell Biology, NCI, Bethesda, MD*
- Utpal P. Davé, MD, *Vanderbilt-Ingram Cancer Center, Vanderbilt University, Nashville, TN*
- Lauren Ehrlich, PhD, *The University of Texas at Austin, Austin, TX*
- Christian Hinrichs, MD, *Genitourinary Malignancies Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Aravind Iyer, PhD, *Protein and Genome Evolution Research Group, NLM/NCBI, Bethesda, MD*
- Park Jung-Hyun, PhD, *Experimental Immunology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Renaud Lesourne, PhD, *INSERM, Toulouse, France*
- Dorian McGavern, PhD, *Viral Immunology and Intravital Imaging Section, NINDS, Bethesda, MD*
- Keiko Ozato, PhD, *Section on Molecular Genetics of Immunity, NICHD, Bethesda, MD*
- Rita Perlingeiro, PhD, *University of Minnesota Medical School, Minneapolis, MN*
- Karl Pfeifer, PhD, *Section on Epigenetics, NICHD, Bethesda, MD*
- Alfred Singer, MD, *Experimental Immunology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Nevil Singh, MD, *University of Maryland School of Medicine, Baltimore, MD*
- Naomi Taylor, MD, PhD, *Pediatric Oncology Branch, Center for Cancer Research, NCI, Bethesda, MD*
- Keji Zhao, PhD, *Laboratory of Epigenome Biology, NHLBI, Bethesda, MD*
- Juan Carlos Zúñiga-Pflücker, PhD, *University of Toronto, Toronto, Canada*

**Contact**

For more information, email *lovep@mail.nih.gov* or visit *https://www.nichd.nih.gov/research/atNICHDI.Investigators/love.*
The Arms Race between Transposable Elements and KRAB-ZFPs and its Impact on Mammals

At the NICHD, our central mission is to ensure that every human is born healthy. Despite much progress in understanding the many ways the mother interacts with the fetus during development, we still know little about the molecular changes that promoted the emergence of placental mammals from our egg-laying relatives over 100 million years ago, nor about those mechanisms that continue to drive phenotypic differences amongst mammals. One attractive hypothesis is that retroviruses and their endogenization into the genomes of our ancestors played an important role in eutherian evolution, by providing protein-coding genes such as syncytins (derived from retroviral env genes that cause cell fusions in placental trophoblasts) and novel gene-regulatory nodes that altered expression networks, to allow for implantation and the emergence and continued evolution of the placenta. Our primary interest is to explore the impact of such endogenous retroviruses (ERVs), which account for about 10% of our genomic DNA, on embryonic development and on the evolution of new traits in mammals. This has led us to examine the rapidly evolving Kruppel-associated box zinc-finger protein (KZFP) family, the single largest family of transcription factors (TFs) in most, if not all, mammalian genomes. Our hypothesis is that KZFP gene expansion and diversification was driven primarily by the constant onslaught of ERVs and other transposable elements (TEs) to the genomes of our ancestors, as a means to transcriptionally repress them. The hypothesis is supported by recent evidence demonstrating that the majority of KZFPs bind to TEs and that TEs and nearby genes are activated in KZFP knockout mice. We will continue to explore the impacts of the TE/KZFP “arms race” on the evolution of mammals. We will also begin a new phase of our research to explore whether KZFPs play broader roles in genome regulation, beyond gene silencing, and how such functions impact mammalian development and evolution.

Kruppel-associated box zinc-finger (ZF) proteins (KRAB-ZFPs) have emerged as candidates that recognize ERVs. KRAB-ZFPs are rapidly evolving transcriptional repressors that emerged in a common ancestor of coelacanths, birds, and tetrapods, and they constitute the largest family of transcription factors in mammals (estimated to be several hundred in mice and humans). Each species has its own unique repertoire of KRAB-ZFPs, with a small number shared by closely
related species and a larger fraction specific to each species. Despite their abundance, little is known about their physiological functions. KRAB-ZFPs consist of an N-terminal KRAB domain that binds to the co-repressor KAP1 and a variable number of C-terminal C2H2 ZF domains that mediate sequence-specific DNA binding. KAP1 directly interacts with the KRAB domain, which recruits the histone methyltransferase (HMT) SETDB1 and heterochromatin protein 1 (HP1) to initiate heterochromatic silencing. Several lines of evidence point to a role for the KRAB-ZFP family in ERV silencing. First, the number of C2H2 ZF genes in mammals correlates with the number of ERVs. Second, the KRAB-ZFP protein ZFP809 was isolated based on its ability to bind to the primer binding site for proline tRNA (PBSpro) of murine leukemia virus (MuLV). Third, deletion of the KRAB-ZFP co-repressors Trim28 or Setdb1 leads to activation of many ERVs. We have therefore begun a systematic interrogation of KRAB-ZFP function as a potential adaptive repression system against ERVs.

We initially focused on ZFP809 as a likely ERV-suppressing KRAB-ZFP, given that it was originally identified as part of a repression complex that recognizes infectious MuLV via direct binding to the 18 nt Primer Binding Site for Proline (PBSpro) sequence. We hypothesized that ZFP809 functions in vivo to repress other ERVs that utilize the PBSpro. Using ChIP-seq of epitope-tagged ZFP809 in embryonic stems cells (ESCs) and embryonic carcinoma (EC) cells, we determined that ZFP809 binds to several sub-classes of ERV elements via the PBSpro. We generated Zfp809 knockout mice to determine whether ZFP809 was required for silencing the ERV element VL30pro. We found that Zfp809 knockout tissues displayed high levels of VL30pro elements and that the targeted elements display an epigenetic shift from repressive epigenetic marks (H3K9me3 and CpG methylation) to active marks (H3K9Ac and CpG hypo-methylation). ZFP809–mediated repression extended to a handful of genes that contained adjacent VL30pro integrations. Furthermore, using a combination of conditional alleles and rescue experiments, we determined that ZFP809 activity was required in development to initiate silencing, but not in somatic cells to maintain silencing. The studies provided the first demonstration of the in vivo requirement of a KRAB-ZFP in the recognition and silencing of ERVs.
FIGURE 2. ZCWPW1 binds to meiotic recombination hotspots in spermatocytes harboring dual H3K4me3 and H3K36me3 marks.

ChIP-seq or Cut & Run was performed with indicated antibodies in mouse spermatocytes, and read pileup is displayed across a region on Chr 4. The pie chart at the right displays percentage of ZCWPW1 peaks that overlap peaks of either H3K4me3, H3K36me3, or both marks.

As a follow-up to our studies on ZFP809, we began a systematic analysis of KRAB-ZFPs using a medium-throughput ChIP-seq screen and functional genomics of KRAB-ZFP clusters and individual KRAB-ZFP genes. Our ChIP-seq data demonstrate that the majority of recently evolved KRAB-ZFP genes interact with and repress distinct and partially overlapping ERVs and other retrotransposons targets. The hypothesis is strongly supported by the distinct ERV reactivation phenotypes we observed in mouse ESC lines lacking one of five of the largest KRAB-ZFP gene clusters. Furthermore, our preliminary evidence suggests that KRAB-ZFP cluster knockout (KO) mice are viable, but have elevated rates of somatic retrotransposition of specific retrotransposon families, providing the first direct genetic link between KRAB-ZFP gene diversification and retrotransposon mobility.

Although our data show that many KRAB-ZFPs repress ERVs, we also found that more ancient KRAB-ZFPs, which emerged in a human/mouse common ancestor, do not bind to or repress ERVs. One such KRAB-ZFP, ZFP568, plays an important role in silencing a key developmental gene that may have played a critical role in the onset of viviparity in mammals. Using ChIP-seq and biochemical assays, we determined that ZFP568 is a direct repressor of a placental-specific isoform of the Igf2 gene called Igf2-P0. Insulin-like growth factor 2 (Igf2) is the major fetal growth hormone in mammals. We demonstrated that loss of Zfp568, which causes gastrulation failure, or mutation of the ZFP568 binding site at the Igf2-P0 promoter, cause inappropriate Igf2-P0 activation. We also showed that the lethality could be rescued by deletion of Igf2. The data highlight the exquisite selectivity by which members of the KRAB-ZFP family repress their targets, and they identify an additional layer of transcriptional control of a key growth factor regulating fetal and placental development. In a follow-up to these studies, we determined that ZFP568 is highly conserved and under purifying selection in eutheria with the exception of human. Human ZNF568 allele variants have lost the ability to bind to and repress Igf2-P0, which may have been driven by the loss of the Igf2-p0 transcript in human placenta. We solved the crystal structure of mouse ZFP568 ZFs bound to the Igf2-P0 binding site, which reveals several non-canonical ZF-DNA contacts, highlighting the ability of individual ZFs to change confirmation depending upon ZF context and DNA structure. The structures also explain how mutations in human ZNF568 alleles disrupt Igf2-P0 interactions, which contain either deleted ZFs or mutations of key ZF-DNA contact residues. Taken together, our studies provide important insights into the evolutionary and structural dynamics of ZF-DNA interactions, which play a key role in regulating mammalian development and evolution.
We began a new exploration of the function and mechanism of PRDM9, the most ancient KRAB-ZFP, which emerged in the jawless fish and which plays a highly specialized role in meiotic recombination (MR). MR generates genetic diversity in sexually reproducing organisms and ensures proper synapsis and segregation of homologous chromosomes in gametes. Errors in MR that lead to mis-segregation of chromosomes are a leading cause of miscarriage and childhood disease. MR is initiated by programmed double-strand breaks (DSBs) in DNA that are distributed non-randomly at thousands of specific 1–2 kb regions called hotspots. In most mammals, hotspots are defined by PRDM9, a protein that contains a rapidly evolving DNA–binding ZF array and a specialized histone methyltransferase activity that catalyzes dual trimethylation marks on histone H3 at lysine 4 and 36 (H3K4me3 and H3K36me3), whose activities are both required for hotspot specification. Prdm9 loss-of-function causes sterility in mice, and PRDM9 mutations have been associated with male infertility in humans. In species lacking Prdm9, including yeast, plants, and birds, hotspots are located in H3K4me3–rich regions at gene promoters. Thus, the emergence of PRDM9 during evolution reshaped the MR landscape by relocating DSBs away from promoters to chromatin sites bound by the rapidly evolving PRDM9, which allowed for rapid interspecies hotspot diversification. We set out to address whether other factors, in addition to PRDM9, are required to “re-engineer” hotspot selection and how the DNA break and repair machinery is recruited to sites marked by PRDM9. We identified the dual histone methylation readers Zcwpw1, which co-evolved with and is tightly co-expressed with Prdm9. Using a mouse model, we found that ZCWPW1 is an essential meiotic recombination factor required for efficient repair of PRDM9–dependent DSBs and pairing homologous chromosomes in males mice. However, ZCWPW1 is not required for the initiation of DSBs at PRDM9 binding sites. Our results indicate that the evolution of a dual histone methylation writer (PRDM9) and reader (ZCWPW1) system in vertebrates remodeled genetic recombination hotspot selection from an ancestral static pattern near genes towards a flexible pattern controlled by the rapidly evolving DNA–binding activity of PRDM9.

Additional Funding
• NICHD Strategic Planning Award: The role of THAP7 in neurodevelopment and intellectual disability
**Publications**


**Collaborators**

- Xiaodong Cheng, PhD, *Emory University, Atlanta, GA*

**Contact**

For more information, email *rodd.macfarlan@nih.gov* or visit *http://macfarlan.nichd.nih.gov*. 
Virulence Mechanisms of Microbial Pathogens

Our main research goal is to define mechanisms by which pathogenic bacteria infect the human host and cause disease, and to investigate whether these mechanisms may be manipulated for preventative and/or therapeutic purposes. As a model organism we use the bacterium *Legionella pneumophila*, the causative agent of a potentially fatal respiratory infection known as Legionnaires’ disease. According to the CDC (Centers for Disease Control), the number of Legionnaires’ disease cases in the U.S. has risen more than four-fold over the past 15 years, making *L. pneumophila* an emerging pathogen of increasing relevance. Contrary to what its name may imply, Legionnaires’ disease occurs in individuals of all ages, including children who receive respiratory therapy, newborns who had recently undergone surgery or under-water birth, and children who are immune-compromised. We are committed to an in-depth analysis of the mechanisms that allow *L. pneumophila* to exploit the human host and cause disease. Insights gained from our studies will ultimately improve our ability to diagnose, prevent, and fight Legionnaires’ disease and related illnesses, thereby contributing to the success of NICHD’s mission.

Within freshwater environments, *L. pneumophila* exists as an intracellular parasite of single-cell organisms know as amoeba. Upon inhalation of contaminated water droplets, *L. pneumophila* enters the lung and is phagocytosed (taken up) by specialized immune cells known as alveolar macrophages (Figure 1). Instead of being degraded by these cells, the pathogen establishes a protective membrane compartment: the *Legionella*-containing vacuole (LCV). Within this intravacuolar niche, *L. pneumophila* can replicate to high numbers before killing the host cell and infecting neighboring cells.

The virulence of *L. pneumophila* relies on the activity of close to 300 proteins, or effectors, that are delivered into the host cytosol by a specialized translocation apparatus called the Dot/Icm type IV secretion system (T4SS) (Figure 1). *L. pneumophila* mutants with a non-functional T4SS are degraded by macrophages, underscoring the importance of the translocated effectors for host-cell manipulation and bacterial virulence.

Our main objective is to obtain detailed mechanistic insight into *L. pneumophila* effectors by investigating their biological role at a cellular level.
FIGURE 1. Intracellular replication cycle of *Legionella pneumophila*

Upon uptake by a macrophage, *L. pneumophila* delivers a large number of effector proteins (*red*) through the Dot/Icm type IV secretion system (T4SS) into the host cytosol. The effectors manipulate signaling and trafficking pathways in order to establish conditions favorable for *L. pneumophila* growth. Eventually, the host cell is lysed, and *L. pneumophila* bacteria infect neighboring cells.

molecular, cellular, and structural level. Knowledge obtained from these studies can help in the development of novel therapeutics aimed at treating or preventing Legionnaires' disease and related illnesses.

**The *Legionella* effector kinase LegK7 hijacks the host Hippo pathway to promote infection.**

Microbial pathogens manipulate host-cell signaling pathways by encoding molecular mimics of protein kinases. Kinases catalyze target phosphorylation by attaching a phosphate group onto side chains of substrate proteins, preferentially serine, threonine, or tyrosine residues, thereby altering the activity, localization, or stability of their substrates. Protein phosphorylation is one of the most abundant and most important post-translational modifications in living cells, and mammals encode hundreds of kinases that target thousands of substrate proteins. To promote infection, many pathogens, including bacteria and eukaryotic parasites, encode effector kinases that are translocated into the host cytosol, where they alter signaling cascades, suggesting that kinase mimicry is a commonly used strategy to exploit host cell function.

Using structural prediction to identify cryptic catalytic domains within *L. pneumophila* effectors, we discovered that the protein LegK7 contains a central domain (residues 183–462) that has folding homology to eukaryotic protein kinases. Using a specific labeling technique, we experimentally confirmed that LegK7 indeed exhibits kinase activity *in vitro*. Upon probing a human protein microarray platform with LegK7, we identified MOB1 as a direct substrate of LegK7 (Figure 2). MOB1 is a key scaffold protein within the Hippo kinase signaling pathway, which, in eukaryotes, controls cell-cycle progression, cell proliferation and differentiation, and apoptosis. In a variety of *in vitro* and cell-based assays, we subsequently showed that LegK7 hijacks the Hippo pathway by molecularly mimicking the host Hippo kinase (MST1 in mammals), the key regulator of pathway activation.
FIGURE 2. Legionella LegK7 manipulates the host cell Hippo signaling pathway.

Cartoon comparing canonical Hippo signaling (left) with signaling by Legionella LegK7 (right). In the canonical Hippo pathway, the Hippo kinase MST1 phosphorylates the N-terminal extension (NTE) of MOB1A on two tyrosine residues, T12 and T35, to promote formation of the MOB1A–LATS1 kinase complex. The activated MOB1A–LATS1 kinase phosphorylates the transcriptional regulators YAP1/TAZ, which results in degradation of YAP1/TAZ and, consequently, expression of genes involved in cell proliferation or differentiation.

During infection, L. pneumophila translocates the effector kinase LegK7 into host cells. Like MST1, LegK7 directly phosphorylates T12 and T35 on the NTE of MOB1A. LegK7 then interacts directly with phosphorylated MOB1A and becomes highly active. Active LegK7 uses MOB1A to recruit downstream substrates, possibly YAP1, for further phosphorylation and manipulates the conserved Hippo pathway to promote infection.

LegK7, like Hippo/MST1, phosphorylates the scaffolding protein MOB1 on two residues, which triggers a signaling cascade, resulting in the degradation of the transcriptional regulators TAZ and YAP1. Transcriptome analysis by RNA-Seq revealed that LegK7–mediated targeting of TAZ and YAP1 alters the transcriptional profile of mammalian macrophages, a key cellular target of L. pneumophila infection. Specifically, genes targeted by the transcription factor PPARg, which is regulated by TAZ, displayed altered expression, and continuous interference with PPARg activity rendered macrophages less permissive to L. pneumophila intracellular growth. Thus, a conserved L. pneumophila effector kinase exploits the Hippo pathway to promote bacterial growth and infection (Figure 2).

Legionella LegK7 exploits the host scaffold protein MOB1A for allostery and substrate phosphorylation.

The finding that LegK7 targets MOB1A and manipulates its function points toward an unexpected role for the Hippo pathway in microbial pathogenesis. Given that LegK7 appears to functionally mimic MST1/2, it became important to more thoroughly investigate this host-pathogen interaction and to determine, at a molecular level, how LegK7 exploits a signaling pathway that is engrained within the developmental program of eukaryotic cells.

Using in vitro kinase reconstitution assays, we showed that MOB1A, in addition to being a substrate of LegK7, functions as an allostERIC activator of its kinase activity. A crystallographic analysis of the LegK7–MOB1A complex revealed that the N-terminal half of LegK7 is structurally similar to eukaryotic protein kinases, and that MOB1A, like the mammalian kinase LATS1/2, directly binds to the LegK7 kinase domain (Figure 3). Substitution of interface residues critical for complex formation abrogated allostERIC activation of LegK7 both in vitro and within cells and diminished MOB1A phosphorylation. We also provided evidence that LegK7 forms a complex
with MOB1A in order to use its N-terminal extension as a binding platform for the recruitment of downstream substrates, so that their phosphorylation can occur more efficiently.

Together, our findings suggest that LegK7 has evolved into a functional chimera of MST1/2 and LATS, and that it combines features from both of the core kinases of the Hippo pathway in order to exploit the downstream signaling cascade. We propose that, by using this strategy, *L. pneumophila* has developed the ability to survive within a wide range of diverse host species, including humans.

**A multiplex CRISPR interference tool for virulence gene interrogation in an intracellular pathogen**

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas gene editing technologies have recently arisen as a mechanism for both fast and targeted gene manipulation in a variety of systems. In the simplest CRISPR-Cas system, Type II, only a single protein known as Cas9 is required for crRNA–guided DNA cleavage, making it the most developed genetic tool. In the absence of target cleavage, catalytically inactive dCas9 imposes transcriptional gene repression by sterically precluding RNA polymerase activity at a given gene to which it was directed by CRISPR (cr)RNAs. This gene silencing technology, referred to as CRISPR interference (CRISPRi), has been employed in various bacterial species to interrogate genes, mostly individually or in pairs. To our knowledge, no group has fully exploited or probed the natural multiplex capability of CRISPR repeat/spacer arrays as a gene silencing tool in bacterial systems.

We thus developed a multiplex (MC) CRISPRi platform in the pathogen *L. pneumophila* capable of silencing up to ten genes simultaneously (Figure 4). We provided proof-of-concept that this novel platform can be used not only during growth in axenic media but also during macrophage infection, where it reproduced known intracellular growth phenotypes. Importantly, by placing the crRNA–encoding spacer in positions further downstream within the array, the degree of gene silencing was titratable. In contrast, when combined with a boxA element, our 10-plex CRISPR array had the potential to silence up to ten unique genes simultaneously (Figure 4), making it a powerful tool to study even synergistic genetic interactions.
Looking forward, the multiplex CRISPRi approach we developed holds the promise of one day probing functional overlap amongst the hundreds of *L. pneumophila* effectors. Not only can genes be silenced in bulk groups during infection, but the mobility of our single plasmid based CRISPRi platform allows for easy transfer of MC constructs into a variety of *Legionella* mutant strain backgrounds to directly assess redundancy, presuming they have been equipped with a copy of dCas9.

**Structural insight into the membrane-targeting domain of the *Legionella* deAMPylase SidD**

AMPylation, the post-translational modification with adenosine monophosphate (AMP), is catalyzed by effector proteins from a variety of pathogens. *L. pneumophila* is thus far the only known pathogen that, in addition to encoding an AMPylase (SidM), also encodes a deAMPylase, called SidD, that reverses SidM–mediated AMPylation of the host-cell vesicle transport GTPase Rab1; deAMPylation is catalyzed by the N-terminal phosphatase-like domain of SidD. The structure of the C-terminal domain and its biological role had remained unclear.

We determined the crystal structure of full length SidD, including the previously uncharacterized C-terminal domain (Figure 5). We found that the C-terminal domain assumed a novel fold not yet described in other proteins, which is critical for membrane localization of SidD within host cells. Notably, the localization depended on two structural elements within the C-terminal domain: a flexible loop composed of hydrophobic amino acid residues, which represents a general membrane-targeting determinant; and a two-helix bundle at the C-terminal end that functions as the specificity determinant. Deletion of either of these elements interfered with the correct localization of SidD within mammalian cells. Moreover, we found that, during growth in mouse macrophages, a *L. pneumophila* strain producing SidD without the loop phenocopied a *L. pneumophila* strain producing no SidD at all, thus revealing that membrane targeting of SidD via its C-terminal domain is a critical prerequisite for its ability to catalyze Rab1 deAMPylation during *L. pneumophila* infection. Future studies will reveal whether additional effectors from *L. pneumophila* or related pathogens use a similar membrane-targeting strategy within host cells.
FIGURE 5. SidD membrane localization requires the C-terminal domain.

A. Domain organization of SidD with the N-terminal domain (NTD, blue) and C-terminal domain (CTD, magenta). The hydrophobic loop (dashed line) is unstructured, indicative of intrinsic flexibility. Numbers indicate amino acid positions.

B. Intracellular localization of CTD variants. Transiently transfected COS-1 cells producing either GFP (control) or GFP-CTD variants were fixed and stained using an antibody directed against the Golgi marker protein giantin (middle). Merged images of SidD proteins (green) and giantin (red). Scale bar, 10 μm.

C. CTD(322-450) localizes to mitochondria membranes. Transiently transfected COS-1 cells coproducing GFP-CTD(322-450) and Mito-RFP (a mitochondria marker) were chemically fixed, and the fluorescence signal was examined by confocal microscopy. Arrowheads indicate the position of membranes magnified in the insets. Scale bar: 10 μm.

Publications

1. Lee PC, Machner MP. The Legionella effector kinase LegK7 hijacks the host Hippo pathway to promote infection. Cell Host Microbe 2018 24:429-438.


Collaborators

- Miroslaw Cygler, PhD, University of Saskatchewan College of Medicine, Saskatoon, Canada
- Robert Heinzen, PhD, Coxiella Pathogenesis Section, Rocky Mountain Laboratories, NIAID, Hamilton, MT
- Aitor Hierro, PhD, CIC bioGUNE Institute, Bilbao, Spain
- Michal Jarnik, PhD, Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD
- Anton Simeonov, PhD, Division of Pre-Clinical Innovation, NCATS, Bethesda, MD
- Chad Williamson, PhD, Advanced Microscope Facility, NICHD, Bethesda, MD

Contact

For more information, email macherm@mail.nih.gov or visit http://machnerlab.nichd.nih.gov.
We are interested in tRNAs and mRNAs as well as some of their interacting proteins and how the pathways involved in their biogenesis, maturation, and metabolism intersect with processes critical to cell proliferation, growth, and development during health and disease. One focus is the synthesis of tRNAs by RNA polymerase III (RNAP III), as well as their post-transcriptional processing and ‘handling’ by the eukaryotic RNA-binding protein La. The protein was discovered because it is a target of autoantibodies in patients who suffer from, is integral to an autoimmune process that leads to, and is diagnostic of Sjögren’s syndrome, systemic lupus erythematosus (SLE), and neonatal lupus. Autoimmunity to La occurs by a complex, as yet incompletely understood mechanism, and the protein is sometimes referred to as the La autoantigen. Critical to its normal essential function, the conserved La protein contains multiple RNA-binding motifs and subcellular trafficking elements, and associates with noncoding (nc) RNAs, mostly in the nucleus, as well as with mRNAs in the cytoplasm. In the nucleus, La binds to the 3′ oligo(U) motif common to all RNAP III transcripts as discrete small RNPs (ribonucleoproteins) and functions by protecting its most abundant ligands, the nascent precursor tRNAs, from 3′ exonucleolytic digestion and by serving as a chaperone to prevent their misfolding. Although the major products of RNAP III are the tRNAs, it also synthesizes 5S rRNA and some other essential noncoding RNAs (ncRNAs) involved in fundamental processes necessary for translating the genetic information in mRNA during protein synthesis.

Our investigations also include specific post-transcriptional modifications of tRNAs that impact their metabolism and function during translation by cytoplasmic and mitochondrial ribosomes. We also study La–related protein-4 (LARP4), which, in contrast to La protein, is predominantly cytoplasmic at steady state, and interacts mostly with mRNAs rather than ncRNAs. However, similar to La, LARP4 interacts with the 3′ end regions of its RNA ligands, in this case the mRNA poly(A) tails (PATs), and contributes to their stability/metabolism and translation. Genome-wide mRNA-Seq and analysis of PATs indicate that LARP4 interacts with a large number of mRNAs and promotes their stability. Such analyses of mice whose LARP4 gene is disrupted reveal shorter PATs, whereas over-expression of LARP4 leads to PAT lengthening. Studies on the mechanisms by which mRNA PAT
metabolism and mRNA stability are linked suggest that LARP4 affects nascent PAT metabolism. Mechanistic studies are under way.

In summary, we strive to understand the structure-function relationship, genetics, cell- and molecular biology of the La protein, LARP4, the tRNA-modification enzymes tRNA isopentenyltransferase TRIT1 and Trm1, and their contributions to growth and development. We use genetics, cell and structural biology, and biochemistry in model systems that include yeast, human and mouse tissue culture cells, and gene-altered mice.

Data from our lab suggest that levels of cytoplasmic tRNAs may regulate translation-mediated decay of LARP4 mRNA and LARP4 levels, which in turn promotes stabilization of mRNAs encoding ribosomal proteins. Tumor suppressors and oncogenes mediate deregulation of tRNA production by RNAP III, collectively contributing to the increased translational capacity required for proliferation during growth and development and of cancer cells. However, tRNA levels or their abundance alone do not account for their differential nor their regulated activity. tRNAs are the most heavily and the most diversely modified molecules in cells, and some of the modifications can control or regulate their codon-specific translation activity. As alluded to above, we study some of the key modifications that affect tRNA translational activity during mRNA decoding.

The tRNA-modification enzyme Trm1 synthesizes dimethyl-guanosine-26 (m^2,2G26), which resides at the top of the anticodon stem of several tRNAs, whereas TRIT1 adds an isopentenyl group onto adenine-37 in the anticodon loop of certain tRNA (i^6A37) molecules. We showed that each of these modifications can activate their tRNAs for translation in codon-specific assays. We also examine effects of TRIT1 on tRNA activity during mammalian development, using TRIT-gene-altered mice as a model system to understand how a deficiency in this enzyme leads to childhood mitochondrial dysfunction and metabolic disease. We complement our studies by investigating how differences in the copy numbers of tRNA genes can affect how the genetic code is deciphered via use of secondary information in the genetic code. In humans, active tRNA genes number more than 300, and are distributed on all chromosomes, many residing in clusters, the loci of which can vary, as do the loci of individual tRNA genes. A system we use to examine codon-specific effects of loss of the i^6A37 modification on a specific tRNA is the fission yeast Schizosaccharomyces pombe, in which human TRIT1 can complement the phenotype caused by deletion of the homologous enzyme Tit1. We also use the S. pombe system to study La and pol III, in which the human La protein can complement the phenotype caused by deletion of the fission-yeast La-homolog Sla1.

One theme of our work and that of others is that differential expression of tRNAs occurs in a tissue- and temporal-specific manner and, together with their differential modifications, controls mRNA decoding in
Activities of RNA polymerase III (RNAP III) and associated factors

The RNAP III multisubunit enzyme complex consists of 17 subunits, several with homology to subunits of RNAPs I and II. The transcription factor TFIIIC, composed of six subunits, binds to A- and B-box promoters (promoter elements of tRNA genes) and recruits TFIIIB to direct RNAP III to the correct start site. TFIIIB–RNAP III complexes appear highly stable and demonstrate great productivity in supporting the many cycles of initiation, termination, and re-initiation necessary to produce the more than tenfold molar excess of tRNAs relative to ribosomes that is required to drive translation during growth and development. In contrast to all other multisubunit RNA polymerases, termination and re-initiation by RNAP III (also known as Pol III) are functionally if not physically linked. Our laboratory has developed methods for \textit{in vivo} and biochemical studies to examine the unique mechanisms used by RNAP III. Hereditary mutations in RNAP III cause hypomyelinating leukodysplasia, as well as defects in innate immunity. In addition to its being essential for cell proliferation, RNAP III is also linked to aging.

Transcription termination delineates 3' ends of gene transcripts, prevents otherwise runaway RNAP from intruding into downstream genes and regulatory elements, and enables release of the RNAP for recycling. While other RNAPs require complex \textit{cis} signals and/or accessory factors to accomplish these activities, eukaryotic RNAP III does so autonomously with high efficiency and precision at a simple oligo(dT) stretch of 5–6 bp. A basis for this high-density \textit{cis} information is that both the template and non-template strands of the RNAP III terminator carry distinct signals for different stages of termination. High-density \textit{cis} information is a feature of the RNAP III system that is also reflected in the dual functionalities of the tRNA promoters as both DNA and RNA elements. Furthermore, the TFIIF–like RNAP III subunit C37 is required for this function of the non-template strand signal. Our results reveal the RNAP III terminator to be an information-rich control element. While the template strand promotes destabilization via a weak oligo(rU:dA) hybrid, the non-template strand provides distinct sequence-specific destabilizing information through interactions with the C37 subunit.

Control of the differential abundance or activity of tRNAs can be an important determinant of gene regulation. RNAP III synthesizes all tRNAs in eukaryotes, and its derepression is associated with cancer. Maf1 is a conserved general repressor of RNAP III under the control of TOR (target of rapamycin), which acts to integrate transcriptional output and protein-synthetic demand toward metabolic economy. We used tRNA-HydroSeq (hydrolysis-based tRNA sequencing) to document that little change occurred in the relative levels of different tRNAs in \textit{maf1} mutated cells. By contrast, the efficiency of \textit{N2,N2-dimethyl G26 (m^{2,2}G26)} modification on certain tRNAs was reduced in response to \textit{maf1} deletion and associated with anti-suppression, which we validated by other methods. Overexpression of Trm1, which produces \textit{m^{2,2}G26}, reversed \textit{maf1} anti-suppression. The model that emerges is that competition by elevated tRNA levels in \textit{maf1-delta} cells leads to \textit{m^{2,2}G26} hypomodification resulting from limiting Trm1, thus reducing the activity of suppressor tRNA\textit{SerUCa} (UCa is the anticodon).
for serine) and accounting for anti-suppression. Consistent with this, RNAP III mutations associated with hypomyelinating leukodystrophy reduce tRNA transcription, increase m\textsuperscript{2,2}G26 efficiency, and reverse anti-suppression. Extending this more broadly, a reduction in tRNA synthesis by treatment with rapamycin leads to increased m\textsuperscript{2,2}G26 modification, a response that is conserved among highly divergent yeasts and human cells [Reference 6].

The ability of RNAP III to efficiently recycle from termination to re-initiation is critical for abundant tRNA production during cellular proliferation, development, and cancer. We used two tRNA-mediated suppression systems to screen for Rpc1 (subunit of RNAP III) mutants with gain- and loss-of-termination phenotypes in Schizosaccharomyces pombe. We mapped 122 point mutation mutants to a recently solved 3.9 Å structure of the yeast RNAP III elongation complex (EC); they cluster in the active-center bridge helix and trigger loop, as well as in the pore and funnel formation, the latter indicating involvement in termination of the RNA-cleavage domain of the C11 subunit of RNAP III. Biochemical kinetic and genetic data indicate that mutants with the RT (readthrough) phenotype synthesize more RNA than wild-type cells and, surprisingly, more than can be accounted for by the mutants' increased elongation rate. Importantly, similar mutations in spontaneous cancer suggest this as an unforeseen mechanism of RNAP III activation in disease.

The role of La-related protein-4 (LARP4) in poly(A)-mediated mRNA stabilization

Ubiquitous in eukaryotes, La proteins are involved in two broad functions: first, metabolism of a wide variety of precursor tRNAs and other small nuclear RNAs by association with these RNAs' common UUU-3′ OH–transcription termination elements; and second, translation of specific subsets of mRNAs, such as those containing 5' IRES (internal ribosome entry site) motifs. LARP4 emerged later in evolution, and we found it to be an mRNA-associated cytoplasmic factor associated with poly(A)–binding protein C1 (PABPC1, PABP). LARP4 uses two regions to bind to PABPC1. We showed that the N-terminal domain (NTD, amino acids 1-286) of LARP4, consisting of an N-terminal region (NTR, amino acids 1-111) followed by two tandem RNA-binding motifs known as an 'La module' (111–285), exhibits preferential binding to poly(A). The NTR contains a unique PAM2w motif that binds to the MLLE (a peptide-binding domain) of PABP. The group of our collaborator Maria Conte showed that the N-terminal region (NTR) itself is responsible for most of the poly(A) binding and that, moreover, this involves conserved residues unique to the PAM2w of LARP4. The La module is flanked by a different motif on each side that independently interact with PABP. LARP4 is controlled at the level of mRNA stability: one level of control is by an A+U-rich element (ARE) in its 3′ UTR via interactions with the protein tristetraproline (TTP), the latter of which is regulated in mammals by tumor necrosis factor alpha (TNFa); a second level of control was found for the LARP4 mRNA–coding sequence in an unusual group of synonymous codons with poor match to cellular tRNA levels [Reference 1]. The LARP4 protein controls the metabolism/homeostasis and translation of heterologous mRNAs by affecting their poly(A) tail length. Working with researchers in the NICHD Molecular Genomics Core facility, we developed a single-molecule, high-throughput nucleotide-resolution poly(A)-tail sequencing method referred to as SM-PAT-Seq, which yielded insights into LARP4 function and mechanism. LARP4 is a global factor involved in mRNA poly(A) length homeostasis and appears to effect mRNA stabilization by opposing the action of deadenylases when poly(A) tails are short.

Fission yeast as a model system for the study of tRNA metabolism and function in translation

More than 20 years ago, we began developing, refining, and advancing a tRNA–mediated suppression (TMS)
system in *S. pombe*, which provides a red-white phenotypic real-time assay that can be used to investigate various aspects of tRNA biogenesis, maturation, and metabolism of tRNAs *in vivo*. In *S. pombe*, the human La protein can replace the tRNA-processing/maturation function of Sla1p, the *S. pombe* equivalent of the La protein. Moreover, in *S. pombe*, human La is faithfully phosphorylated on Ser-366 by protein kinase CKII, the same enzyme that phosphorylates Ser-366 in human cells, and this phosphorylation event promotes pre-tRNA processing. We use the system to study transcription by RNAP III, post-transcriptional processing, and tRNA modifications by conserved enzymes that produce tRNA isopentenyl-adenosine-37 and dimethylguanosine-26.

**tRNAs, codon use, and mRNA metabolism in growth and development**

A major interest of ours is to decipher what we refer to as ‘secondary information’ in the genetic code, information that is derived from mRNAs’ biased use of synonymous codons. This can produce a layer of information beyond the amino acid sequence of a protein; i.e., in addition to providing the template for the sequence of a protein, the use of certain synonymous codons can also produce additional biochemical effects, which we refer to as ‘secondary information.’ The effects can be related to ribosome pausing, which can affect protein folding, or to alterations in the stability of the mRNA. Other types of secondary information can also be encoded in synonymous codons; for example, sets of mRNAs that share similar patterns of synonymous codon bias are similarly sensitive to tRNAs with the same anticodon modification and exhibit similar patterns of efficiency of translation elongation. The components of the secondary information system are the tRNA pool, the tRNA-modification enzymes, and the codon bias distribution among the mRNAs. We recently found that synonymous codon use by the human LARP4 mRNA is a key determinant in the control of the expression levels of its mRNA and protein, and that increases in otherwise limiting tRNAs that are cognate to these codons increase LARP4 production. This in turn activates LARP4 to promote a net increase in the poly(A) tail length of heterologous mRNAs, including those that encode ribosomal protein subunits [Reference 1], which may be important because ribosome production is regulated during growth and development, and the potential circuit involving LARP4 control by tRNA could be an important point of control.

**Publications**

Collaborators

- James R. Iben, PhD, Molecular Genomics Core, NICHD, Bethesda, MD
- Maria R. Conte, PhD, King’s College, University of London, London, United Kingdom
- Steven Coon, PhD, Molecular Genomics Core, NICHD, Bethesda, MD

Contact

For more information, email maraiar@mail.nih.gov or visit http://maraialab.nichd.nih.gov.
Immune Activation and Viral Pathogenesis

The general goal of the Section for Intercellular Interactions is to understand the tissue-pathogenic mechanisms of human pathogens and the role of cytokines in such processes. We focused on the pathogenesis of human immunodeficiency virus 1 (HIV-1) and on its co-pathogens, in particular herpesviruses. We found that HIV-1 virions may carry the cytokine TGF beta to their cell target, probably facilitating HIV-1 infection. Also, the cytokine spectrum in semen appears to be an important determinant of HIV-1 transmission in vivo. Soluble and extracellular vesicle (EV)–associated cytokines are linked to the development of cardiovascular diseases, in particular of atherosclerosis triggered by HIV-1 infection. We also launched a project on SARS CoV-2 pathogenesis in human lung tissue ex vivo.

Macrophage–derived HIV-1 carries TGF-beta.

HIV-1 virions released by productively infected cells, predominantly T cells and macrophages, contain not only virus-encoded proteins but also some cellular proteins. The functions of the majority of cell-encoded molecules incorporated into virions, unlike those of virus-encoded molecules, are mostly not known, even though they may play an important role in HIV infection.

We focused on HIV-1 virions, produced by macrophages, given that in vivo these cells are long-term producers of viruses and probably dominate in the late stages of HIV disease. We used the single-virion analysis (“flow virometry”) that was developed in our laboratory several years ago and found that some of the HIV-1 virions produced by macrophages, along with viral antigens, incorporate into the viral membrane a macrophage scavenger receptor CD36.

We found that some CD36+ virions isolated from plasma of HIV–infected individuals are associated with transforming growth factor beta (TGF-beta), one of the key cytokines regulating immune responses. TGF-beta was found only on CD36+ virions derived from macrophages, not on CD27+ virions derived from T cells. TGF-beta binds to CD36 via thrombospondin-1(TSP-1), the main activator of TGF-beta. We proved that TGF-beta bound to macrophage-derived HIV remains bioactive, as shown using reporter cells.
Thus, HIV-1 not only induces general immune responses to infection but already carries cytokines that may affect the immune response, specifically in target cells. Viral-associated cytokines, together with cytokines produced by infected tissues, may determine a significant aspect of HIV-1 infection, including transmission, in particular sexual transmission.

Our work for demonstrated the first time that HIV virions can be associated with cytokines, namely TGF-beta.

**Cytokines in HIV-1 transmission**

A stated above, viral-associated cytokines, together with cytokines produced by infected tissues, may determine HIV-1 infection, in particular its sexual transmission. To address this question, we compared the cytokine/chemokine profiles in the blood and semen of source partners to investigate whether the profiles are associated with HIV transmission in the men's recent sexual partners, who either became infected with a phylogenetically linked HIV strain (where the putative source is referred to as a transmitter) or did not acquire HIV (where the putative source partner is referred to as a non-transmitter). Viral transmission was confirmed by phylogenetic linkage (HIV pol). Using the multivariate statistical technique of partial least square discriminant analysis, we compared cytokine profiles of transmitters with those of non-transmitters. We found that the cytokine profiles of transmitters and non-transmitters were statistically different in semen but not in blood, suggesting that blood cytokines were not a good predictor of HIV transmission in our cohort mostly of men who have sex with men (MSM). There was a trend of higher concentrations of pro-inflammatory cytokines (RANTES, IL-18, IL-6, and GRO-alpha) in the blood of transmitters than in that of non-transmitters, but the overall difference was not statically significant.

In contrast to blood, the cytokine profiles in the semen of transmitters and non-transmitters were different, and the difference was highly significant. The finding reflects the fact that semen is not just a vector for HIV, but a complex milieu that carries pro- and antiviral factors that may facilitate or inhibit HIV transmission. The unique cytokine profile in the semen of the transmitter group suggests that seminal cytokines are likely to be an important determinant of HIV transmission. The differences in seminal cytokine profiles associated with transmission or non-transmission of HIV from infected MSM to their partners was still evident after the cytokine concentrations were adjusted for semen HIV RNA and cytomegalovirus (CMV) DNA.

We found no cytokines in semen that were associated with a higher risk of transmission. Rather, we found an associated group of cytokines: IFN-gamma, IL-13, M-CSF, IL-17, and GM-CSF. All cytokines except IL-13 were found in higher concentrations in non-transmitters than in transmitters. IFN-gamma and IL-13, the two cytokines with the highest differences, belong to different functional helper T cell type groups: IFN-gamma is the archetypal cytokine of the helper T cells type 1 (Th1) cells and supports cytotoxic T-cell responses, while IL-13 (together with IL-4) is secreted by helper T cells type 2 (Th2) cells, which counter-regulate Th1 responses and activate humoral immunity. A Th1/Th2 imbalance was originally described as a critical step in the etiology of HIV infection. Th1–associated cytokines have protective effects against HIV infection, whereas a shift towards an augmented humoral Th2 response may be detrimental and lead to the progression of HIV infection to acquired immunodeficiency syndrome.

Our study provides the first correlations between seminal cytokines in the transmitting partner and HIV transmission. The seminal cytokine spectrum is a contributing determinant of sexual HIV transmission, thus providing new directions for the development of strategies aimed at preventing HIV transmission.
Cytokines in HIV-1–triggered atherosclerosis

Dysregulation of cytokines in HIV-1–uninfected individuals is associated with T cell migration into atherosclerotic plaques. We showed that cytokines are released in both soluble and extracellular vesicle (EV)–associated forms. We investigated the expression and clustering of soluble and EV–associated cytokines in patients with ST-elevation myocardial infarction (STEMI). We found that several clustered cytokines were expressed almost exclusively in STEMI patients and were released in a coordinated fashion. Identification of such cytokine clusters permits investigation of their distinct contributions to STEMI. The cytokine pattern in STEMI patients resembles that triggered by viruses.

Although the etiological factor for atherosclerosis is not known, one such case is established: long-term HIV infection. In collaboration with a Case Western University team, we investigated the mechanisms by which cytokines trigger T cell recruitment to, and activation within, plaques. We found elevated expression of CX3CL1 (also known as fractalkine) in atherosclerotic plaques. The cytokine is known for eliciting its adhesive and migratory functions by interacting with the chemokine receptor CX3CR1, which we found to be upregulated on CD8+ T cells. Thus, endothelial cell–derived CX3CL1 may direct the migration of CX3CR1–expressing CD8+ T cells to the activated endothelium. These findings, together the observation that another elevated cytokine, IL-15, a strong activator of CD8+ T cells, induced expression of cytolytic molecules by the migrated CD8+ T cells, provide a mechanism for the damage caused to endothelia in the aortas of SIV- or SHIV–infected rhesus macaques.

In confirmation that a similar mechanism of atherosclerosis operates in humans, we found increased number of CD8+ T cells in atherosclerotic vessels of HIV–uninfected individuals. CD8+ T cells that accumulate in human atherosclerotic plaques have an activated, resident phenotype consistent with in vivo IL-15 and CX3CL1 exposure. Together these observations provide a novel model for CD8+ T cell involvement in atherosclerosis: CX3CL1 and IL-15 operate in tandem within the vascular endothelium to promote infiltration by activated CX3CR1+ memory CD8+ T cells, leading to atherosclerosis progression. Such processes are further promoted by the common HIV-1 co-pathogen CMV. The new results thus constitute newly discovered mechanisms of T cell infiltration into atherosclerotic plaques linked to cytokine disbalance.

SARS CoV-2 tissue pathogenesis

SARS CoV-2 infects lung epithelia, triggering severe pathology. Investigation of the mechanisms of SARS CoV-2 tissue pathogenesis and development of antiviral strategies in particular require a deep understanding of SARS CoV-2 pathogenesis in the context of human tissue systems under laboratory-controlled conditions. We developed such a system. Human lung tissue is dissected into 2mm3 blocks and cultured at the air-liquid interface. The histology of the blocks revealed well-preserved structural elements, including alveoli with epithelial cells. Flow cytometry of cells from these blocks confirmed their viability and expression of the ACE-2 receptor. To prove that the lung explants can be infected with SARS CoV-2, we used SARS CoV-2 pseudoviruses expressing the SARS-CoV-2 S protein that mediates cell infection with the virus. This pseudovirus encodes eGFP, thus making the infected cells fluoresce and allowing us to monitor viral infection. We tested the SARS CoV-2 pseudoviruses for infectivity on cell lines expressing ACE-2 and on lung tissues, and they were proven to be infectious. Development of human lung-tissue ex vivo pseudoviruses now allows us to characterize the main target cells of SARS CoV-2 with high-parameter flow cytometry.
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Publications


Collaborators

- Michael Bukrinsky, MD, PhD, *George Washington University, Washington, DC*
- Leonid Chernomordik, PhD, *Section on Membrane Biology, NICHD, Bethesda, MD*
- Sara Gianella Weibel, MD, *University of California San Diego, La Jolla, CA*
- Michael Freeman, PhD, *Case Western University, Cleveland, OH*
- Sergey Kochetkov, PhD, *Engelhard Institute of Molecular Biology, Moscow, Russia*
- Michael Lederman, MD, *Case Western University, Cleveland, OH*
- Roberto Romero-Galue, MD, DMedSci, *Perinatology Research Branch, NICHD, Detroit, MI*
- Yoel Sadovsky, MD, *Magee-Womens Research Institute, University of Pittsburgh, Pittsburgh, PA*
- Alexandr Shpekotor, MD, *Moscow Medical University, Moscow, Russia*
- Elena Vasilieva, MD, *Moscow Medical University, Moscow, Russia*
- Beatrice Vitali, PhD, *Università di Bologna, Bologna, Italy*

Contact

For more information, email *margolis@helix.nih.gov* or visit *http://irp.nih.gov/pi/leonid-margolis*.
In an integrated program of laboratory and clinical investigation, we study the molecular biology of the heritable connective tissue disorders collectively known as osteogenesis imperfecta (OI). Our objective is to elucidate the mechanisms by which the primary gene defect causes skeletal fragility and other connective tissue symptoms and then to apply this knowledge to the treatment of children with these conditions. We identified several key genes causing recessive and X-linked OI. Discoveries of defects in collagen modification have generated a new paradigm for OI as a collagen-related disorder of matrix. We established that structural defects in collagen cause dominant OI, while deficiency of proteins that interact with collagen for folding, post-translational modification, or processing cause recessive OI. Our challenge now is to understand the cellular and biochemical mechanisms of recessive OI. We also generated a knock-in murine model for OI with a classical collagen mutation as well as a murine model for recessive type IX OI and X-linked type XVIII OI, and we are using these models to study disease pathogenesis and the skeletal matrix of OI, the effects of pharmacological therapies, and approaches to gene therapy. Our clinical studies involve predominantly children with types III and IV OI, who form a longitudinal study group enrolled in age-appropriate clinical protocols for the treatment of their condition.

We are also investigating melorheostosis, a very rare bone dysostosis, which is characterized by radiographic patterns of either “dripping candle wax” or endosteal bone overgrowth. We recently identified mosaic mutations in the oncogene MAP2K1 as the cause “dripping candle wax” melorheostosis and somatic mutations in SMAD3 as causative of endosteal melorheostosis. In each gene, the causative mutations occur at a hot spot and result in gain-of-function. We are now developing animal models for studies of melorheostosis pathophysiology and treatment.

Mechanism of rare forms of osteogenesis imperfecta
Recessive null mutations in SERPINF1, which encodes pigment epithelium–derived factor (PEDF), cause OI type VI. PEDF is well-known as a potent anti-angiogenic factor. Type VI OI patients have no
serum PEDF, elevated alkaline phosphatase (ALPL) as children, and bone histology with broad unmineralized osteoid and a fish-scale pattern. However, we identified a patient with severe atypical type VI OI, whose osteoblasts displayed minimal secretion of PEDF, but whose SERPINF1 sequences were normal despite typical type VI OI bone histology. Surprisingly, exome sequencing on this proband and family members yielded a de novo mutation in IFITM5 (the gene encoding interferon-induced transmembrane protein 5, which is mutated in type V OI) in one proband allele, causing a p.S40L substitution in the intracellular domain of BRIL, the encoded protein. The IFITM5 transcript and BRIL were normal in proband fibroblasts and osteoblasts. SERPINF1 expression and PEDF secretion were reduced in proband osteoblasts. In contrast, osteoblasts from a typical case of type V OI have elevated SERPINF1 expression and PEDF secretion during osteoblast differentiation. Together, the data suggest that BRIL and PEDF have a relationship that connects the genes for types V and VI OI and their roles in bone mineralization.

The endoplasmic reticulum (ER)–resident procollagen 3-hydroxylation complex is responsible for the 3-hydroxylation of type I collagen alpha1(I) chains. Deficiency of components of the collagen P3H (prolyl 3-hydroxylase) complex causes recessive OI. For type VIII OI, we investigated bone and osteoblasts. Collagen has near-absent 3-hydroxylation from both bone and dermis, demonstrating that P3H1 is the unique enzyme responsible for collagen 3-hydroxylation. Bone histomorphometry revealed patches of increased osteoid, although the overall osteoid surface was normal. Quantitative backscattered electron imaging (qBEI) showed increased mineralization of cortical and trabecular bone, as in other OI types. However, the proportion of bone with low mineralization was higher in type VIII bone than in type VII, consistent with patchy osteoid occurring only in type VIII.

The third member of the complex, cyclophilin B (CyPB), encoded by PPIB, is an ER–resident peptidyl-prolyl cis-trans isomerase (PPIase). CyPB is the major PPIase catalyzing collagen folding. We characterized the first patient with deficiency in PPIB, which causes recessively inherited type IX OI. Our group generated a Ppib knock-out (KO) mouse model that recapitulates the type IX OI phenotype. Intracellular collagen folding occurs more slowly in CyPB null cells, supporting the enzyme’s role as the rate-limiting step of folding. However, treatment of KO cells with the cyclophilin inhibitor cyclosporin A caused further delay in folding, providing support for the existence of a further collagen PPIase. We found that CyPB supports collagen lysyl hydroxylase 1 (LH1) activity, demonstrating significantly reduced hydroxylation of the helical crosslinking residue K87, which directly affects both the extent and type of collagen intermolecular crosslinks in bone. However, CyPB deficiency results in increased hydroxylation at telopeptide crosslinking sites in tendon, with moderate increase in glycosylation. In our recent collaboration with Mitsuo Yamauchi and colleagues [Reference 2], we explored the role of CyPB in post-translational modifications of collagen in skin. As in bone, hydroxylation of collagen crosslinking sites was almost absent in mutant mice, and the key cross-linking residue alpha1(I)K87 was under-glycosylated. Absence of CyPB led to the occurrence of two novel types of collagen crosslinks that are not present in normal skin. Atomic force microscopy showed that this was associated with a lower nano-indentation modulus in KO than in normal skin. The studies underscore the tissue-dependent effects of CyPB, which have common effects on cross-linking and mechanical properties.

In collaboration with Vorasuk Shotelersuk and Cecilia Giunta, we identified a new OI-causative gene on the X-chromosome. This is the first type of OI with X-linked inheritance, and it causes a moderate to severe bone dysplasia with pre- and postnatal fractures of ribs and long bone, bowing of long bones, low bone density, kyphoscoliosis and pectal deformities, and short stature. Affected individuals have missense mutations...
in *MBTPS2*, which encodes the protein S2P. S2P is a transmembrane protein in the Golgi and is a critical component of regulated intramembrane proteolysis (RIP). In RIP, regulatory proteins are transported from the ER membrane to the Golgi in times of cell stress or sterol depletion, where they are sequentially cleaved by S1P/S2P to release activated N-terminal fragments, which enter the nucleus and activate gene transcription. Mutant S2P protein is stable but has impaired RIP functioning, with deficient cleavage of the ER–stress transducers OASIS, ATF6, and SREBP. Furthermore, hydroxylation of the collagen residue K87 is reduced by half in proband bone, consistent with reduced lysyl hydroxylase in proband osteoblasts. Reduced collagen crosslinks presumably undermine bone strength. The mutations in *MBTPS2* demonstrate that RIP plays a fundamental role in bone development.

**C-propeptide cleavage–site mutations increase bone mineralization.**

Type I procollagen is processed to mature collagen by the removal of both N- and C-terminal propeptides. The C-propeptide is cleaved at the Ala-Asp peptide bond between the telopeptide and the C-propeptide of each chain by procollagen C-proteinase (also known BMP-1 or bone-morphometric protein). Probands with substitutions at any of the four cleavage-site residues have a high-bone-mass form of OI, first reported by our lab in collaboration with Katarina Lindahl. The patients have elevated bone-density DEXA Z-scores and, in bone histology, patchy unmineralized osteoid. The processing of the C-propeptide from collagen secreted by proband cells is delayed. Using bone mineralization density distribution (BMDD), we investigated mineralization to show that, in the alpha2(I) cleavage site mutation, the bone had a uniformly higher mineral density, while in the alpha1(I) mutation, the average mineral density was markedly heterogeneous, with areas of either very high or low bone density.

To investigate the role of the C-propeptide in bone mineralization and developmental progression, we developed a knock-in murine model with a *COL1A1* (the gene encoding the pro-alpha1 chain of type 1 collagen) cleavage site mutation. Bone collagen fibrils showed a “barbed-wire” appearance consistent with the presence of the processing intermediate pC-collagen, which was detected in extracts of bone from mutant mice, and with impaired collagen processing *in vitro*. Impaired C-propeptide processing affects skeletal size and biomechanics. The mice are small, and their femora exhibit extreme brittleness on mechanical testing, as well as reduced fracture load. BMDD measurement on their femora show significantly higher mineralization than in wild-type (WT) mice, which continues to increase in HBM (high bone mass) mice even after WT mineralization plateaus at 6 months. PINP and TRAP, serum markers of bone remodeling, are significantly elevated in HBM mice. Osteocyte density is reduced but lacunar area is increased.

**Mutations in the COL1A1 C-propeptide**

The C-propeptide of type I collagen (*COL1A1* C-propeptide) is processed after collagen is secreted from the cell and before it is incorporated into matrix. Interestingly, mutations in the C-propeptide are present in about 6% of OI patients. Our investigation of the biochemical consequences of C-propeptide mutations in comparison with collagen helical mutations revealed both intra- and extracellular differences [Reference 3]. Immunofluorescence microscopy indicated that procollagen with C-propeptide defects was mis-localized to the ER lumen, in contrast to the ER membrane localization of normal procollagen and to helical mutations. Second, although the mutations were not located in the processing site itself, pericellular cleavage of the C-propeptide was defective in both pericellular processing assays and in cleavage assays with purified C-proteinase, consequences that are expected to contribute to abnormal osteoblast differentiation and matrix function, respectively.
Insights from the Brtl mouse model for OI

The Brtl mouse model for OI, generated by our lab, is a knock-in mouse that contains a Gly349Cys substitution in the alpha1(I) collagen chain. Brtl was modeled on a type IV OI child and accurately reproduces features of type IV OI. Brtl has provided important insights into OI mechanism and treatment.

We collaborated with Kenneth Kozloff’s group to investigate a potential anabolic therapy, sclerostin antibody (Scl-AB), which stimulates osteoblasts via the canonical Wnt pathway. Scl-AB stimulated bone formation in young Brtl mice and increased bone mass and load-to-fracture. Treatment with Scl-AB caused no detrimental change in Brtl bone material properties. Nano-indentation studies indicated unchanged mineralization, unlike the hyper-mineralization induced by bisphosphonate treatment. In addition, Scl-AB was successfully anabolic in adult Brtl mice, and may be a therapy for adult patients who have fewer treatment options. Because Scl-AB is a short-acting drug, we recently investigated sequential Scl-AB/bisphosphonate treatment. The study showed that administration of a single dose of bisphosphonate after cessation of Scl-AB treatment preserved anabolic gains from Scl-AB. Alternatively, a single low dose of bisphosphonate concurrent with Scl-AB treatment facilitated the anabolic action of Scl-AB by increasing availability of trabecular surfaces for new bone formation. Because a lifelong deficiency of sclerostin leads to patterns of excessive cranial bone growth and nerve compression, we undertook dimensional and volumetric measurements of the skulls of Brtl mice treated with Scl-AB. Treated mice showed calvarial thickening but minimal effects on cranial morphology and anatomic landmarks. Narrowing of vascular but not neural foramina was seen.

Brlt mice provided important information on the cytoskeletal organization in OI osteoblasts and their potential role in phenotypic variability. We observed abnormal cytoskeletal organization involving vimentin, strathmin, and coflin-1 in lethal pups. Decreased vimentin (an intermediate filament) can lead to cytoskeletal collapse, and increased strathmin (a regulatory factor that promotes microtubular disassembly) and coflin-1 (an inducer of actin depolymerization) work in concert to disrupt cytoskeletal cellular functions. The alterations affected osteoblast proliferation, collagen deposition, integrin, and TGF-beta signaling. The data suggest that cytoskeletal elements present novel OI treatment targets. Another potential novel treatment may be 4-PBA, a chemical chaperone. When the drug is used to treat OI cells, it enhances autophagy, as opposed to apoptosis, of the cells and stimulates protein secretion. Interestingly, the enhanced protein secretion reflects a broad range of cellular proteins rather than simply the retained mutant collagen and relieves the ER stress along the PERK pathway.

Two basic insights have emerged from Brtl studies. The first concerns hyper-mineralization of OI bone, which was previously thought to be a passive process. Altered levels for osteocyte transcripts involved in bone mineralization, such as Dmp1 and Sost, demonstrated, however, the presence of an actively directed component. We used acoustic transmission microscopy to characterize the properties of Brtl cortical bone. The periodically oriented collagen organization in periosteal cortex of Brtl bone was markedly lower than in normal bone. Young’s modulus and ER sound velocity were significantly increased in Brtl cortex. The data demonstrate that the mutant collagen of Brtl mice affects the mechanical behavior of bone predominantly in the endosteal region by altering collagen orientation. Second, the osteoclast is important for the OI phenotype, with elevated numbers of osteoclasts. Co-culture experiments with Brtl and wild-type (WT) mesenchymal stem cells (MSCs) and osteoclast precursors yielded elevated osteoclast numbers from WT or Brtl precursors grown with Brtl MSCs, but not with WT MSCs. The results indicate that an osteoblast product is necessary to elevate osteoclast numbers.
Natural history and bisphosphonate treatment of children with types III and IV OI

We recently published the cardiopulmonary aspects of our natural history study on types III and IV OI. Longitudinal evaluations were completed in 23 children with type III OI and 23 children with type IV OI, who had pulmonary function tests every 1–2 years. Compared with size-matched children, our patients showed a significant decline over time in pulmonary function, including in lung volumes and flow rates. The decline was worse in the 36 children with scoliosis (average curve 25 degrees) but also occurred in 20 participants without scoliosis, who had declining function with restrictive disease, suggesting that the pulmonary dysfunction of OI is attributable to a primary defect in the lung related to structurally abnormal collagen. The studies are important because pulmonary issues are the most prevalent cause of morbidity and mortality in OI. Affected individuals should seek anticipatory evaluation and treatment.

Previously, OI–specific growth curves were not available, despite the fact that short stature is one of the cardinal features of OI. We assembled longitudinal length, weight, head circumference, and body mass index (BMI) data on 100 children with types III and IV OI with known mutations in type I collagen, to generate sex- and type-specific growth curves for OI. We examined effects of gender, OI type, and pathogenic variant, using multilevel modeling, and we constructed OI–specific centile curves, using a generalized additive model for location, scale, and shape (GAMLSS). The data show that gender and OI type, but not the collagen chain in which the causative mutation is located, have significant effects on height in OI. Boys are taller than girls, and type IV OI boys and girls are taller than type III. In both genders, length curves for types III and IV OI overlap, and the type IV 95th centile curve overlaps the lower U.S. Centers for Disease Control and Prevention (CDC) curves for the general population. A pubertal growth spurt is generally absent or blunted in types III/IV OI. The BMI 50th and 95th centile curves are distinctly shifted above respective CDC curves in both genders. Weight differs by OI type, but not by gender or mutant collagen chain. Interestingly, head circumference does not differ by gender, OI type, or collagen mutation. Imposition of OI height curves on standard CDC curves reveals an overlapping of type III and IV percentiles and the absence of a growth spurt in type III OI. Standard growth curves for OI will be of great value to primary caregivers and families and will provide a baseline for treatment trials.

Our randomized controlled trial of bisphosphonate in children with types III and IV OI was the first randomized bisphosphonate trial for OI in the United States. It examined direct skeletal and secondary gains reported in uncontrolled trials. For skeletal outcomes, we found increased BMD (bone mineral density) Z-scores and improved vertebral geometry. We noted that vertebral BMD improvement tapered off after two years' treatment. Our treatment group did not experience fewer long-bone fractures, coinciding with the lack of improvement or equivocal improvement in fractures in other controlled trials. This controlled trial did not support the claims for improvement in ambulation level, lower-extremity strength, or alleviation of pain, suggesting these were placebo effects in observational trials. Our current recommendation is for treatment for 2–3 years, with subsequent follow-up of bone status. We are now engaged in a dose-comparison trial, comparing the dose from our first trial with a lower dose, achieved by increasing the cycle interval at the same dose/kg/cycle. Our preliminary analysis indicates that OI children obtain comparable benefits from lower and higher doses of pamidronate.

Melorheostosis: genetic and clinical delineation

Melorheostosis is a very rare sporadic bone dysostosis that is characterized by metabolically active bone in the appendicular skeleton, which leads to asymmetric bone overgrowth, seen radiographically as “dripping candle
wax,” functional impairment, and pain. Skin overlying the bone lesion sometimes has a hyperpigmented, vascular lesion. Given that attempts to identify germline mutations causing melorheostosis were unsuccessful, we proposed somatic mutations. Our collaborative team (with Tim Bhattacharyya, Richard Siegel, and Nadja Fratzl-Zelman) was the first to look directly at bone samples. Fifteen patients with melorheostosis had paired biopsies of both affected and contralateral unaffected bone.

DNA extracted from each patient’s two bone samples was subjected to whole-exome sequencing (WES); sequences from each individual patient were compared, and secondarily compared among the set of patients. We identified two genes causing somatic mutations in melorheostotic lesions. Each gene was associated with one of the radiographic forms of melorheostosis, and the bone lesions had distinct histology and mechanism along the TGFß pathway. In affected but not unaffected bone or blood, eight of the 15 patients had somatic mutations for MAP2K1, located in two adjacent residues of the negative regulatory domain and would be expected to increase MEK1 activity. Increased MAPK activity along the non-canonical TGFβ pathway leads to increased phosphorylation and activation of ERK1/2, accounting for the mosaic pattern of increased p-ERK1/2 in osteoblasts on immunohistochemistry of affected bone. Osteoblasts cultured from affected bone constitute two populations with distinct p-ERK1/2 levels by flow cytometry, enhanced ERK1/2 activation, and elevated cell proliferation. However, the MAP2K1 mutations inhibit BMP2–mediated osteoblast mineralization and differentiation in vitro, underlying the markedly increased osteoid detected in affected bone histology. Our data show that the MAP2K1 oncogene is important in human bone formation and implicate MAP2K1 inhibition as a potential treatment avenue for melorheostosis.

Four patients were determined to have causative somatic mutations in SMAD3 [Reference 4], a component of the canonical TGFβ pathway. SMAD3 phosphorylation was increased in affected bone, and downstream target genes of TGFβ signaling had increased expression. The mutations were associated with an endosteal radiographic pattern. Cultured osteoblasts from affected bone exhibited decreased proliferation in vitro, increased expression of osteoblast differentiation markers, and increased mineralization. However, the constitutive activation of the SMAD3 dampened the activity of BMP2, because addition of BMP2 to culture media decreased osteoblast differentiation and mineralization in vitro. Bone lesions with SMAD3 mosaicism did not show increased cellularity or osteoid accumulation and were more highly mineralized.

Melorheostotic bone from both MAP2K1–positive and SMAD3–positive patients showed two zones of distinct morphology. In MAP2K1–positive melorheostosis [Reference 5], the inner osteonal zone is intensively remodeled and has increased osteoid, which is covered by a zone containing compact multi-layered lamellae. The remodeling zone has lower than normal bone mineralization and a high porosity, reflecting increased tissue vascularity. The lamellar portion is less mineralized than the remodeling zone, indicating a younger tissue age. Nano-indentation was not increased in the lamellar zone, indicating that the surgical hardness of this bone reflects its lamellar structure. We propose that the genetically induced deterioration of bone microarchitecture in the remodeling zone triggers a periosteal reaction.

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Publications


Collaborators

- Patricia Becerra, PhD, *Laboratory of Retinal Cell and Molecular Biology*, NEI, Bethesda, MD
- Timothy Bhattacharyya, MD, *Clinical and Investigative Orthopedics Surgery Unit*, NIAMS, Bethesda, MD
- Anne De Paepe, MD PhD, *Universitair Ziekenhuis Gent*, Ghent, Belgium
- David Eyre, PhD, *University of Washington*, Seattle, WA
- Antonella Forlino, PhD, *Università degli Studi di Pavia*, Pavia, Italy
- Nadja Fratzl-Zelman, PhD, *Ludwig Boltzmann-Institut für Osteologie, Hanusch Krankenhaus der WGGK und Unfallkrankenhaus Medling*, Vienna, Austria
- Cecilia Giunta, PhD, *Kinderspital Zürich*, Zürich, Switzerland
- Wolfgang Högler, MD, DSc, FRCPCH, *Birmingham Children’s Hospital NHS Foundation Trust*, Birmingham, United Kingdom
- Kenneth Kozloff, PhD, *University of Michigan*, Ann Arbor, MI
- Sergey Leikin, PhD, *Section on Physical Biochemistry*, NICHD, Bethesda, MD
- Katarina Lindahl, MD, *Uppsala Universitet*, Uppsala, Sweden
- Scott Paul, MD, *Rehabilitation Medicine*, NIH Clinical Center, Bethesda, MD
- Cathleen L. Raggio, MD, *Weill Medical College of Cornell University*, New York, NY
- Vorasuk Shotelersuk, MD, FABMG, *King Chulalongkorn Memorial Hospital*, Bangkok, Thailand
- Richard Siegel, MD, PhD, *Autoimmunity Branch*, NIAMS, Bethesda, MD
- Mitsuo Yamauchi, PhD, *University of North Carolina*, Chapel Hill, NC
- Joshua Zimmerberg, MD, PhD, *Section on Integrative Biophysics*, NICHD, Bethesda, MD

Contact

For more information, email marinij@mail.nih.gov or visit https://irp.nih.gov/pi/joan-marini.
Hippocampal Interneurons and Their Role in the Control of Network Excitability

Cortical and hippocampal GABAergic inhibitory interneurons (INs) are “tailor-made” to control cellular and network excitability by providing synaptic and extrasynaptic input to their downstream targets via GABA$_A$ and GABA$_B$ receptors. The axons of this diverse cell population make local, short-range projections (although some subpopulations project their axons over considerable distances) and release the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) onto a variety of targets. Distinct cohorts of INs regulate sub- and supra-threshold intrinsic conductances, regulate Na$^+$- and Ca$^{2+}$-dependent action-potential generation, modulate synaptic transmission and plasticity, and pace both local- and long-range large-scale synchronous oscillatory activity. An increasing appreciation of the roles played by INs in several neural-circuit disorders, such as epilepsy, stroke, Alzheimer’s disease, and schizophrenia, has seen this important cell type take center stage in cortical circuit research. With almost 30 years of interest in this cell type, the main objectives of the lab have been to understand: (1) the developmental trajectories taken by specific cohorts of INs as they populate the nascent hippocampus and cortex; (2) how ionic and synaptic mechanisms regulate the activity of both local circuit GABAergic INs and principal neurons (PN) at the level of small, well defined networks; and (3) how perturbations in their function alter the cortical network in several neural circuit disorders. To this end, we use a variety of electrophysiological, imaging, optogenetic, immunohistochemical, biochemical, molecular, and genetic approaches with both wild-type and transgenic animals.

Paradoxical network excitation by glutamate release from VGlut3$^+$ CCK–containing hippocampal GABAergic interneurons

Several neuronal subtypes utilize more than one classical neurotransmitter, in violation of Dale’s principle. Molecular identification of vesicular glutamate transporter 3– and cholecystokinin-expressing cortical interneurons (CCK$^+$VGlut3$^+$INTs) has prompted speculation of GABA/glutamate co-release from these cells for almost two decades despite a lack of direct evidence. We now have unequivocally demonstrated CCK$^+$VGlut3$^+$INT–mediated GABA/glutamate co-transmission onto principal cells in adult mice using paired whole-cell electrophysiological recording and optogenetic
FIGURE 1. Hippocampal GABAergic terminals

Three major types of GABAergic terminal are shown in the hippocampal dentate gyrus (red, blue, and green punch). The black negative space is the cell bodies of principal cells innervated by the GABAergic terminals.

approaches. Although under normal conditions GABAergic inhibition dominates CCK-VGluT3+INT signaling, glutamatergic signaling becomes predominant when glutamate decarboxylase (GAD) function is compromised. CCK-VGluT3+INTs exhibit surprising anatomical diversity, comprising subsets of all known dendrite-targeting CCK+ interneurons in addition to the expected basket cells, and their extensive circuit innervation profoundly dampens circuit excitability under normal conditions. However, in contexts where the glutamatergic phenotype of CCK-VGluT3+INTs is amplified, they promote paradoxical network hyperexcitability, which may be relevant to disorders involving GAD dysfunction, such as schizophrenia or vitamin B6 deficiency. Such co-release is expected to impart the unique computational properties to CCK-VGluT3+INTS, differentiating them from CCKBCs lacking VGluT3, which are otherwise morphologically/electrophysiologically indistinguishable. Indeed, CCK-VGluT3+INTS exhibit unique participation in, and regulation of, hippocampal network oscillations and place-cell (cells that act as a cognitive representation of a specific location in space) spatial information coding.

Activity-dependent tuning of intrinsic excitability in mouse and human neurogliaform inhibitory interneurons

The ability to modulate the efficacy of synaptic communication between neurons constitutes an essential property critical for normal brain function. Animal models have proved invaluable in revealing a wealth of
diverse cellular mechanisms underlying varied plasticity modes. However, to what extent these processes are mirrored in humans is largely uncharted, thus questioning their relevance to human circuit function. In this study, led by Ramesh Chittajallu, we focus on a novel type of inhibitory interneuron, the neurogliaform cells (NGFC), which possess specialized physiological features enabling them to impart a widespread inhibitory influence on neural activity. We demonstrated that this prominent neuronal subtype, embedded in both mouse and human neural circuits, undergoes remarkably similar activity-dependent modulation, manifesting as epochs of enhanced intrinsic excitability. In principle, these evolutionary conserved plasticity routes likely tune the extent of neurogliaform cell-mediated inhibition, thus constituting canonical circuit mechanisms underlying human cognitive processing and behavior.

Comparative studies such as the one described here are vital for determining to what extent circuit features gleaned from experimental animal models are relevant in humans. Particularly, we uncovered a previously undescribed evolutionarily conserved mechanism that manifests as a short-term enhancement in the propensity of depolarizing inputs to evoke action potential output. Remarkably, amongst the varied IN located in superficial regions of cortical and hippocampal microcircuits, NGFCs in both species were found to be privileged with regard expression of these forms of plasticity. Together, our data reveal the presence of cellular mechanisms that result in modulation of intrinsic excitability of NGFCs that represent circuit motifs important for human brain function.

Translatome analysis using conditional ribosomal tagging in GABAergic interneurons and other sparse cell types
GABAergic interneurons comprise a small but diverse subset of neurons in the mammalian brain, which tightly regulate neuronal circuit maturation and information flow and, ultimately, behavior. Because of their centrality in the etiology of numerous neurological disorders, examining the molecular architecture of these neurons under various physiological scenarios has piqued the interest of the broader neuroscience community. The last few years have seen an explosion in next-generation sequencing (NGS) approaches aimed at identifying genetic and state-dependent subtypes in neuronal diversity. Although several approaches are employed to address neuronal molecular diversity, ribosomal tagging has emerged at the forefront of identifying the translatomes of neuronal subtypes, an approach that primarily relies on Cre recombinase-driven expression of hemagglutinin A (HA)-tagged RiboTag mice exclusively in the neuronal subtype of interest. This allows the immunoprecipitation of cell type–specific, ribosome-engaged mRNA, expressed both in the soma and the neuronal processes, for targeted quantitative real-time PCR (qRT-PCR) or high-throughput RNA sequencing analyses. Vivek Mahadevan and Areg Peltekian described the typical technical caveats associated with successful application of the RiboTag technique for analyzing GABAergic interneurons, and in theory other sparse cell types, in the central nervous system.

AMPA receptor deletion in developing MGE–derived hippocampal interneurons causes a redistribution of excitatory synapses and attenuates postnatal network oscillatory activity.
Inhibitory interneurons derived from the medial ganglionic eminence (MGE) represent the largest cohort of GABAergic neurons in the hippocampus. In the CA1 hippocampus, excitatory synapses onto these cells comprise GluA2-lacking, calcium-permeable AMPA receptors (AMPARs). Although synaptic transmission is not established until early in their postnatal life, AMPARs are expressed early in development; their role is however enigmatic. Former postdoctoral fellow Gülcan Akgül genetically deleted GluA1, GluA2, and GluA3 selectively
from MGE-derived interneurons early in development, using the Nkx2.1-cre mouse line. We observed that the number of MGE-derived interneurons was preserved in the mature hippocampus despite early elimination of AMPARs, which resulted in an over 90% reduction in spontaneous excitatory synaptic activity. Of particular interest is the observation that excitatory synaptic sites were shifted from dendritic to somatic locations while maintaining a normal NMDAR content. The developmental switch of NMDARs from GluN2B–containing early in development to GluN2A–containing on maturation was similarly unperturbed despite the loss of AMPARs.

The oscillatory activity of early network-driven giant depolarizing potentials was compromised in early postnatal days, as were both feedforward and feedback inhibition onto pyramidal neurons, underscoring the importance of glutamatergic drive onto MGE-derived interneurons for hippocampal circuit function.

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**Publications**

**Collaborators**
- Jordan Dimidschstein, PhD, *The Broad Institute, Cambridge, MA*
- Gordon Fishell, PhD, *The Broad Institute, Cambridge, MA*
- Timothy J. Petros, PhD, *Unit on Cellular and Molecular Neurodevelopment, NICHD, Bethesda, MD*
- Kareem Zaghloul, MD, PhD, *Functional and Restorative Neurosurgery Unit, NINDS, Bethesda MD*

**Contact**
For more information, email mcbainc@mail.nih.gov or visit https://www.nichd.nih.gov/research/atNICHD/Investigators/mcbain or https://dir.ninds.nih.gov/Faculty/Profile/chris-mcbain.html.
Pathophysiology, Genetics, and Treatment of Congenital Adrenal Hyperplasia

In its most severe classic form, congenital adrenal hyperplasia (CAH) is a life-threatening, rare orphan disease that is part of the neonatal screen performed in all 50 U.S. states [Reference 1]. In its mildest non-classical form, CAH is one of the most common autosomal recessive diseases and may be a common cause of female infertility. Our intramural NIH research program strives to elucidate the pathophysiology and genetics of CAH, thus facilitating the development of new approaches to the diagnosis, evaluation, and treatment of the disease. We are conducting the largest ever Natural History Study of CAH, with over 450 patients enrolled. We were the first to identify adrenaline deficiency as a new hormonal imbalance in CAH and the first to report in CAH smaller-than-normal amygdala, the emotion regulator of the brain, providing insight into hormonal effects on the brain. We found that approximately 10 to 15 percent of patients with CAH owing to 21-hydroxylase deficiency have a contiguous gene deletion syndrome resulting in connective tissue dysplasia, hypermobility-type Ehlers-Danlos syndrome, which represents a novel phenotype named CAH-X. Central to our work is the study of new treatments, including a long-term trial testing sex hormone blockade in children, and novel ways of replacing cortisol, aimed at mimicking the normal circadian rhythm of cortisol secretion. The NIH Clinical Center is the ideal venue in which to carry out such studies and is one of the few places in the world that facilitates the conduct of long-term studies of rare diseases.

Adrenal crisis prevention

Patients with adrenal insufficiency are at risk for life-threatening, salt-wasting adrenal crises. Management of illness episodes aims to prevent adrenal crises. We evaluated rates of illnesses and associated factors in our large cohort of patients with adrenal insufficiency attributable to congenital adrenal hyperplasia, who were followed prospectively at the NIH Clinical Center and received repeated glucocorticoid stress-dosing education. We performed longitudinal analysis of over 2,200 visits from 156 CAH patients over 23 years [Reference 2]. During childhood, there were more illness episodes and stress dosing than during adulthood; however, more emergency room visits and hospitalizations occurred during adulthood. The most robust predictors of stress dosing were young age, low hydrocortisone dose, and high fludrocortisone dose during childhood, and, during
adulthood, female sex. Gastrointestinal and upper respiratory-tract infections were the two most common precipitating events for adrenal crises and hospitalizations across all ages. Life-threatening adrenal crisis with hypoglycemia occurred in 11 pediatric patients (ages 1.1–11.3 years). Undetectable epinephrine was associated with emergency room visits during childhood and illness episodes during adulthood.

This longitudinal assessment of illnesses, glucocorticoid stress-dosing practices, and illness sequelae in patients with adrenal insufficiency from CAH resulted in recommendations to revise age-appropriate glucocorticoid stress-dosing guidelines to include more frequent glucocorticoid dosing and frequent intake of simple and complex carbohydrates. Our new age-appropriate guidelines aim to reduce adrenal crises and prevent hypoglycemia, particularly in children. Our suggestions were incorporated into the Endocrine Society Clinical Practice Guideline for Congenital Adrenal Hyperplasia.

Genotype-phenotype studies of CAH-X

CAH is most commonly caused by 21-hydroxylase deficiency. The gene encoding 21-hydroxylase, CYP21A2, and a highly homologous pseudogene, CYP21A1P, map to the short arm of chromosome 6 within the human leukocyte antigen histocompatibility complex. The deleterious sequence in the CYP21A1P pseudogene can be transferred to the CYP21A2 functional gene by homologous recombination, and such events produce common mutations that account for approximately 95% of all CYP21A2 disease-causing mutations. Of the common mutations, approximately 30% are large deletions. The TNXB gene, encoding tenascin-X, an extracellular matrix protein that is highly expressed in connective tissue, and a highly homologous pseudogene, TNXA, flank CYP21A2 and CYP21A1P, respectively. Autosomal recessive tenascin X deficiency was described as a cause of Ehlers-Danlos syndrome in 2001. We hypothesized that deletions of CYP21A2 might commonly extend into the TNXB gene, and we have been studying this phenomenon in our Natural History Study.

The first evaluation of the potential clinical implications of TNXB heterozygosity in CAH patients was performed in our Natural History Study of CAH (www.ClinicalTrials.gov Identifier No. NCT00250159) at the NIH Clinical Center. In 2013, we prospectively studied 193 consecutive unrelated patients with CAH with clinical evaluations for manifestations of Ehlers-Danlos syndrome and genetic evaluations for TNXB mutations. Heterozygosity for a TNXB deletion was present in 7% of CAH patients; such CAH patients were more likely than age-and sex-matched CAH patients with normal TNXB to have joint hypermobility, chronic joint pain, multiple joint dislocations, and a structural cardiac valve abnormality detected by echocardiography. Six of 13 probands had a cardiac abnormality, including the rare quadricuspid aortic valve, a left ventricular diverticulum, and an elongated anterior mitral valve leaflet. As a result of the study, the term CAH-X was coined to describe the subset of CAH patients who display an Ehlers-Danlos syndrome phenotype resulting from to the monoallelic presence of a CYP21A2 deletion extending into the TNXB gene.

The study of CAH-X has provided insight into the recombination events that occur in the class III region of the major histocompatibility complex (MHC) locus, a region of the genome that is predisposed to genetic recombination and misalignment during meiosis. The majority of deletions generate chimeric CYP21A1P/CYP21A2 genes. Chimeric recombination between TNXB and TNXA also occurs (Figure 1). The recombination event deletes CYP21A2 and therefore represents a CAH disease-causing allele. We described three unique types of TNXA/TNXB chimera (CH): CAH-X CH-1 renders the gene nonfunctional, resulting in reduced dermal and serum TNX expression; CAH-X CH-2 alters protein structure; and CAH-X CH-3 is predicted to reduce protein folding energy. Our laboratory continues to investigate how TNXB contributes to the phenotype of CAH patients.
FIGURE 1. Schematic of CYP21A1P/CYP21A2 and TNXA/TNXB chimeric genes

Formation of chimeric genes occurs as a result of misalignment of homologous genes during meiosis. Active genes are in solid colors; pseudogenes are in grey and are framed with the color of the corresponding functional gene. Representative chimeric genes are shown. In total, there are nine known CYP21A1P/CYP21A2 chimeras (CH-1 to CH-9), and we identified three different types of TNXA/TNXB chimeras (CAH-X CH-1 to CAH-X CH-3) with different junction sites. Approximately 10 percent of patients with CAH owing to 21-hydroxylase deficiency carry at least one TNXA/TNXB chimera, resulting in hypermobility-type Ehlers-Danlos syndrome or CAH-X syndrome.

To date, we have described 24 patients (19 families) with monoallelic CAH-X and three patients with biallelic CAH-X. Approximately 10 to 15 percent of patients with CAH resulting from 21-hydroxylase deficiency are now estimated to be affected by CAH-X. Overall, CAH-X patients have generalized joint hypermobility, subluxations, and chronic arthralgia, and about 25% have cardiac structural abnormalities. Patients with biallelic CAH-X show severe skin hyperextensibility with delayed wound healing and significant joint hypermobility. Other connective-tissue disease manifestations in CAH-X patients include chronic tendonitis and/or bursitis, rectal prolapse, severe gastroesophageal reflux, and cardiac abnormalities. Genetic testing for CAH-X is complex and complicated by pseudogene interference and the large, 70kb size of the TNXB gene. In 2019, we developed a PCR–based, high-throughput, cost-effective assay that accurately identifies CAH-X [Reference 3]. The assay had 100% sensitivity and 99.2% specificity.

The study of CAH-X syndrome provides insight into the complex clinical and genetic characteristics associated with CAH and promises to improve patient outcome through the development of focused medical management aimed at preventing long-term consequences.

New and improved biomarkers of CAH

The diagnosis and management of CAH has been limited by inadequate biomarkers. Several pitfalls have been identified in the use of 17-hydroxyprogesterone, the most commonly used biomarker, for both diagnosis and management. The development of liquid chromatography-tandem mass spectrometry (LC-MS/MS) panels of adrenal steroids has expanded the repertoire of potential new and improved steroid
biomarkers. We found that steroids synthesized with the participation of 11beta-hydroxylase (11-oxygenated C19 steroids) are abundant in patients with CAH resulting from 21-hydroxylase deficiency (Figure 2). With our collaborators Richard Auchus and Adina Turcu, we compared traditional and 11-oxygenated androgens in patients with non-classic (mild) CAH resulting from 21-hydroxylase deficiency and patients with symptoms of hyperandrogenism from other causes. Patients with non-classic CAH present with clinical manifestations of hyperandrogenism, features that are shared with other disorders of androgen excess. In particular, the clinical phenotype of women with non-classic CAH is similar to the more common polycystic ovarian syndrome. The diagnosis of non-classic CAH is based on serum 17-hydroxyprogesterone and usually requires dynamic testing with synthetic ACTH (cosyntropin) testing. We found that 11-oxygenated C19 steroids are disproportionately elevated compared with conventional androgens in non-classic CAH, and steroid panels can accurately diagnose non-classic CAH in unstimulated blood tests [Reference 4]. We continue to explore the utility of these newly described steroids in the diagnosis and management of CAH.

Novel treatment approaches: sex steroid blockade and inhibition

As an alternative approach to the treatment of CAH, the effects of elevated androgen and estrogen could be prevented through the use of sex steroid blockade. Short-term (two-year) administration of an antiandrogen and aromatase inhibitor and reduced hydrocortisone was shown to normalize linear growth rate and bone maturation. A prospective long-term randomized parallel study to adult height of an antiandrogen (flutamide) and an aromatase inhibitor (letrozole), and reduced hydrocortisone dose vs. conventional treatment is near completion. The main outcome is adult height, and we will compare data between the treatment groups. The goal of this novel treatment approach is to normalize the growth and development of children with CAH and, ultimately, to determine whether the treatment regimen is effective in improving the growth of children with CAH. The Clinical Center is the ideal place to carry out such a long-term study of a rare disease.
Since the inception of our study of peripheral blockade of sex hormones using an antiandrogen and aromatase inhibitor, new and improved drugs that block sex steroids have been developed. In collaboration with the group of Perrin White, we are studying abiraterone, an irreversible inhibitor of 17a-hydroxylase, a key enzyme required for testosterone synthesis, in a multicenter Phase 1/2 study in prepubescent children (NCT 02574910).

In 2020, we reported for the first time the use of nevanimibe, an orally administered ACAT1/sterol O-acyltransferase 1 (SOAT1) inhibitor, as adjuvant therapy for CAH [Reference 5]. The enzyme is the gatekeeper for the esterification of cholesterol, a necessary step for adrenocortical steroid biosynthesis. This proof-of-concept study showed that short-term (two-week) use of nevanimibe at various doses reduced 17-hydroxyprogesterone, a biomarker of adrenal androgen production. Larger studies of longer duration are needed, but use of the drug, which selectively inhibits adrenal cortex function, might reduce androgen excess and thus allow for lower glucocorticoid dosing in CAH.

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**Publications**


**Collaborators**

- Richard J. Auchus, MD, PhD, *University of Michigan, Ann Arbor, MI*
- Veronica Gomez-Lobo, MD, *Children’s National Health System, Washington, DC*
- James Marko, MD, *Radiology and Imaging Sciences, NIH Clinical Center, Bethesda, MD*
- Martha Quezado, MD, *Laboratory of Pathology, NCI, Bethesda, MD*
- Richard J. Ross, MD, *University of Sheffield, Sheffield, United Kingdom*
- Ninet Sinaii, PhD, MPH, *Biostatistics and Clinical Epidemiology Service, NIH Clinical Center, Bethesda, MD*
Contact
For more information, email dmerke@nih.gov or visit https://irp.nih.gov/pi/deborah-merke.
Childhood Neurodegenerative Lysosomal Storage Disorders

The Section on Developmental Genetics conducts both basic and translational research into a group of the most common childhood neurodegenerative lysosomal storage disorders (LSDs), called neuronal ceroid lipofuscinoses (NCLs), commonly known as Batten disease. The diseases affect mostly children and there is no curative treatment for any of the NCLs. Mutations in at least 14 different genes (called CLNs) underlie various forms of NCLs. The CLN1, CLN2, CLN5, CLN10, and CLN13 genes encode soluble lysosomal enzymes; The CLN4 and CLN14 encode peripherally associated cytoplasmic proteins; the CLN11 encodes progranulin, a protein in the secretory pathway; and several transmembrane proteins with various subcellular localizations are encoded by CLN3, CLN6, CLN7, CLN8, and CLN12. The infantile NCL (INCL), a devastating neurodegenerative LSD, is caused by inactivating mutations in the CLN1 gene, which encodes a lysosomal depalmitoylating enzyme called palmitoyl-protein thioesterase-1 (PPT1).

Currently, the investigations in our laboratory focus on understanding the molecular mechanisms of pathogenesis underlying INCL (CLN1-disease), juvenile NCL (JNCL: CLN3-disease), and congenital NCL (CNCL: CLN10-disease). Interestingly, all NCL types share some common clinical and pathologic features, such as intracellular accumulation of auto-fluorescent material, epileptic seizures, progressive psychomotor decline resulting predominantly from loss of cortical neurons in the cerebrum, neuro-inflammation, visual impairment resulting from retinal degeneration, and shortened lifespan.

Several years ago, we started investigating the INCL, which is caused by inactivating mutations in the CLN1 gene. Notably, numerous proteins in the body, especially in the brain, undergo a reversible post-translational lipid modification called S-palmitoylation (also called S-acylation), whereby a 16-carbon, saturated fatty acid (generally palmitic acid) is attached to specific cysteine residues in polypeptides via thioester linkage. While S-palmitoylation plays important roles in membrane anchorage of soluble proteins, protein-protein interaction, protein trafficking, and protein stability, these lipid-modified proteins must also be depalmitoylated for recycling or degradation and clearance by lysosomal hydrolases. Thus, dynamic S-palmitoylation (palmitoylation-depalmitoylation), like phosphorylation-dephosphorylation, regulates the function of many proteins, especially in the brain. It requires coordinated
action of two types of enzymes with opposing functions. The enzymes that catalyze S-palmitoylation are palmitoyl acyltransferases (PATs), which are zinc-finger proteins with a common DHHC (Asp-His-His-Cys) motif, and they are called ZDHHC PATs or simply ZDHHCs. The mammalian genome encodes a family of 23 ZDHHC PATs.

Similarly, the palmitoyl thioesterases, which depalmitoylate S-acylated proteins, are localized either in the lysosomes like PPT1 or in the cytoplasm like acyl-protein thioesterase-1 (APT1). Recently, a group of protein depalmitoylases called ABHD17 were identified, which catalyze the turnover of N-Ras (a GTP-ase signal-transduction protein).

PPT1 catalyzes the cleavage of thioester linkage of S-palmitoylated proteins, which is vitally important because these lipid-modified proteins are refractory to degradation by lysosomal hydrolases. Thus, PPT1 deficiency leads to lysosomal accumulation of the S-palmitoylated proteins (constituents of ceroid), which has been proposed to be the mechanism of INCL pathogenesis. However, the precise molecular mechanism underlying INCL pathogenesis has remained elusive for more than two decades. Children afflicted with INCL are normal at birth but, by 11 to 18 months of age, exhibit signs of psychomotor retardation. By two years of age, they are completely blind owing to retinal degeneration and, by age four, they manifest no brain activity and remain in a vegetative state for several more years before eventual death. Such grim outcomes underscore the urgent need for the development of rational and effective therapeutic strategies, not only for INCL but also for all NCLs.

The aim of our translational research is to apply the knowledge gained from our basic laboratory investigations to develop novel therapeutic strategies for Batten disease. The results of our earlier investigations on INCL led to a bench-to-bedside clinical trial. Using Cln1-knockout (Cln1–/–) mice, which recapitulate virtually all clinical and pathological features of INCL, we discovered that PPT1 deficiency causes endoplasmic-reticulum (ER) and oxidative stress, which at least in part causes neuronal death by apoptosis. During the past several years, we also delineated a mechanism by which PPT1 deficiency disrupts the recycling of the synaptic vesicle (SV) proteins, which are essential for regenerating fresh SVs to replenish the SV pool size at the nerve terminals to maintain uninterrupted neurotransmission. We also discovered that ER and oxidative stress contribute to neuronal apoptosis and neuro-inflammation in INCL. Further, we found that PPT1 deficiency causes misrouting of the V0a1 subunit of v-ATPase (the proton pump on lysosomal membrane), which dysregulates lysosomal acidification, causing elevated pH and thus adversely affecting lysosomal degradative function.

We also developed a noninvasive method, using MRI and MRS (magnetic resonance spectroscopy), to evaluate the progression of neurodegeneration in Cln1–/– mice. The methods permit repeated evaluation of potential therapeutic agents in treated animals. Application of such methods in our clinical trial with INCL also allowed us to evaluate the progressive decline in brain volume and neurodegeneration. In collaboration with Wadih Zein, we are also conducting studies to determine whether electro-retinography can be used to assess the progressive retinal deterioration in Cln1–/– as well as in Cln1–knock-in (KI) mice, which carry the nonsense mutation in the CLN1 gene commonly found in the INCL patient population in the US. Moreover, we discovered that the blood-brain barrier is disrupted in Cln1–/– mice and that this pathology is ameliorated by treatment with resveratrol, which has antioxidant properties. More recently, we discovered that a nucleophilic small molecule with antioxidant properties, N-(tert-butyl) hydroxylamine (NtBuHA), ameliorates the neurological abnormalities in Cln1–/– mice and extends their lifespan. The compound is currently undergoing preclinical evaluation for the approval of an IND by the FDA. Intriguingly, we discovered that in Cln1–/– mice, the lysosomes contain insufficient amounts of PPT1-protein and PPT1-enzymatic activity, contributing to neuropathology in this disease. These and related studies
Mechanism of dysregulated lysosomal acidification in a mouse model of INCL

In eukaryotic organisms, the lysosome is the primary organelle for intracellular digestion. It contains enzymes that require acidic pH for optimal degradative function. Thus, lysosomal acidification is of fundamental importance in the degradation of macromolecules of intra- and extracellular origin, which are delivered to the lysosome. Moreover, it has been reported that dysregulation of lysosomal acidification contributes to pathogenesis in virtually all LSDs, including NCLs. Furthermore, defective regulation of lysosomal pH has also been reported in common neurodegenerative diseases such as Alzheimer’s and Parkinson’s. However, despite intense studies, the precise mechanism(s) underlying defective lysosomal acidification in these diseases has remained elusive. Lysosomal acidification is regulated by vacuolar ATPase (v-ATPase), a multi-subunit protein complex consisting of the cytosolic V1 sector and the lysosomal membrane-anchored V0-sector. Reversible assembly of V1/V0 sectors on lysosomal membrane maintains functionally active v-ATPase, the proton pump of the cell, which regulates lysosomal acidification.

In contrast to S-palmitoylation, the thioesterases, two of which are cytosolic (APT1 and APT2) and two (PPT1 and PPT2) are localized to the lysosome. Dynamic S-palmitoylation (palmitoylation–depalmitoylation) requires coordinated action of these two groups of enzymes with opposing functions (i.e., ZDHHCs and PPTs), which maintains the steady-state membrane localization and function of numerous important proteins, especially in the brain. By catalyzing depalmitoylation, thioesterases also facilitate recycling or degradation of proteins that undergo S-palmitoylation.

In the present study, we tested a hypothesis that one or more subunits of v-ATPase require S-palmitoylation for endosomal sorting, trafficking and reversible assembly of V1/V0 on lysosomal membrane, which is essential for regulating lysosomal pH, and that Ppt1 deficiency disrupts v-ATPase activity, impairing its proton transport function, thereby dysregulating acidification of lysosomal lumen. Our results show that the lysosomal membrane-anchored V0 sector isoform a1 (V0a1) subunit of v-ATPase indeed undergoes S-palmitoylation, which is required for its sorting and trafficking to the lysosomal membrane. The process appears to be defective in Ppt1-deficient Cln1−/− mice. Notably, we demonstrate that treatment of these mice with a thioesterase (Ppt1)–mimetic small molecule, N-(tert-butyl) hydroxylamine (NtBuHA), restores near-normal v-ATPase activity and rescues the defective lysosomal acidification phenotype. The results demonstrate the potential of NtBuHA as a drug target for INCL.

Palmitoyl–protein thioesterase−1 deficiency dysregulates autophagy in Cln1−/− mice.

Autophagy is an essential cellular degradative process, by which dysfunctional cytoplasmic contents...
are digested by lysosomal hydrolases. There are several autophagic pathways, which include macroautophagy, microautophagy, and chaperone-mediated autophagy. During autophagy, several vesicle fusion events, including those of autophagosome and lysosome, lead to the eventual degradation and clearance of the cargo within a hybrid structure called the autophagolysosome. Several autophagy genes (called Atg) encoding components of the autophagic process are required for the initiation of autophagy. One of these events is marked by the de novo formation of double-membrane structures called phagophores. Two ubiquitin-like conjugation systems involving the Atg5-Atg12-Atg16 complex and phosphatidylethanolamine-conjugated microtubule-associated protein 1 light chain 3 (LC3-II) are required for the initiation of autophagic process. Emerging evidence indicates that dysregulation of autophagy is one of the central pathogenic mechanisms underlying many human diseases including the LSDs.

Emerging evidence indicates that S-palmitoylation plays critical roles in endosomal sorting and trafficking of subcellular proteins to their destinations, which are especially important in the nervous system. In eukaryotic cells, vesicular transport facilitates intracellular proteins to reach their destinations. In this process, a large superfamily of Ras–like GTPases (called Rabs) play pivotal roles in vesicle formation, cargo selection, sorting, transport, and vesicular fusion, all of which are critical for endocytic and autophagic degradation. One of the proteins belonging to the Rab superfamily of GTPases is Rab7. It directly or indirectly performs several important functions in vesicular trafficking and membrane fusion events that occur between early endosome and late endosome/lysosome. Upon interaction with RILP (Rab–interacting lysosomal protein) on late endosomal/lysosomal membrane, Rab7, also known as RAB7A, facilitates the autophagosome-lysosome fusion, generating a hybrid organelle called the autolysosome, which facilitates the degradation of cargo from intracellular sources by lysosomal acid hydrolases. Thus, impaired autophagosome-lysosome fusion is one of the suggested mechanisms for the accumulation of un-degraded cargo in the lysosome leading to the pathogenesis of LSDs. In this study, we sought to determine whether autophagy is dysregulated in INCL, and if so, what might be the underlying mechanism(s).

We found that autophagy is dysregulated in Cln1–/– mice and in postmortem brain tissues from an INCL patient, as well as in cultured INCL fibroblasts. Moreover, Rab7 requires S-palmitoylation for trafficking to the late endosomal/lysosomal membrane, which is dysregulated in Cln1–/– mice. Notably, the defect inhibited the Rab7–RILP interaction, which is essential for Rab7 GTPase activity and required for autophagosome-lysosome fusion. The defect impaired degradative functions of the autolysosome (a hybrid structure arising from the fusion of autophagosome with lysosome), causing lysosomal accumulation of un-degraded cargo, and thus leading to INCL pathogenesis. Importantly, treatment of INCL fibroblasts with NtBuHA, a brain-penetrant, PPT1–mimetic small molecule, ameliorated the defective Rab7–RILP interaction. Our findings reveal a previously unrecognized role of CLN1/PPT1 in autophagy and suggest that thioesterase-mimetic small molecules may ameliorate the dysregulated autophagy, with therapeutic implications for INCL.

Dysregulated crosstalk between lysosomal thioesterase PPT1 and cytosolic thioesterase APT1 contributes to neuro-inflammation in INCL mouse model.

Previously, we reported that the thioesterase APT1 undergoes dynamic S-palmitoylation for shuttling between the cytosol and the plasma membrane, where it depalmitoylates H-Ras, regulating its signaling pathway, which stimulates cell proliferation. While we demonstrated that APT1 catalyzes its own depalmitoylation, the ZDHHC(s) that catalyzes its S-palmitoylation had remained unidentified. We found that ZDHHC5 and ZDHHC23 catalyze...
APT1 S-palmitoylation. Intriguingly, in *Cln1*–/– mice, deficiency of Ppt1 in the lysosome reduced the levels of ZDHHC5 and ZDHHC23. Remarkably, in the brain of these mice decreased ZDHHC5 and ZDHHC23 levels reduced the levels of S-palmitoylated APT1, thereby increasing the level of plasma membrane-localized H-Ras, which activated its signaling pathway stimulating microglia proliferation. The increase in microglia producing inflammatory mediators contributed to neuro-inflammation, thus leading to neurodegeneration. Our results identified ZDHHC5 and ZDHHC23 as the enzymes that catalyze S-palmitoylation of APT1, and revealed a pathway to microglia proliferation and neuro-inflammation in a lysosomal Ppt1-deficient mouse model of INCL. Our results reveal a previously unrecognized pathway to neurodegeneration in INCL and demonstrate that NtBuHA exerts its neuro-protective effects by suppressing the generation of neurotoxic A1 astrocytes in the brain.

**Cln3**–mutations, which underlie JNCL, cause significantly reduced levels of Ppt1 protein and Ppt1 enzyme activity in the lysosome.

Given that intracellular accumulation of ceroid is a characteristic of all NCLs, a common pathogenic link for INCL and JNCL has been suggested. It has been reported that *CLN3* mutations suppress the exit of the cation-independent mannose 6-phosphate receptor (CI-M6PR) from the trans-Golgi network (TGN). CI-M6PR transports soluble proteins such as PPT1 from the TGN to the lysosome. We therefore hypothesized that *CLN3* mutations cause lysosomal PPT1 insufficiency, contributing to JNCL pathogenesis. We found that the lysosomes in *Cln3*–mutant mice, which mimic JNCL, and those in cultured cells from JNCL patients, contain significantly reduced levels of Ppt1 protein and Ppt1 enzyme activity and progressively accumulate auto-fluorescent ceroid. Furthermore, in JNCL fibroblasts, the V0a1 subunit of v-ATPase is mislocalized to the plasma membrane instead of to its normal location on lysosomal membrane. The defect dysregulates lysosomal acidification, as we previously reported in *Cln1*–/– mice. Our findings uncover a previously unrecognized role of *CLN3* in lysosomal homeostasis and suggest that *CLN3* mutations causing lysosomal Ppt1 insufficiency may at least partly contribute to JNCL pathogenesis.

**Defective lysosomal Ca++ homeostasis contributes to impaired autophagy in a mouse model of INCL.**

Autophagy is dysregulated in virtually all LSDs. Previously, we reported that autophagy is dysregulated in *Cln1*–/– mice. However, the precise mechanism underlying the defect has remained elusive. We found that, in *Cln1*–/– mice, lysosomal Ca++ homeostasis is dysregulated owing to reduced levels of the inositol 1, 4, 5-triphosphate receptor type-1 (IP3R1), which contributes to Ca++ transport from the ER to the lysosome. Intriguingly, the transcription factor NFATC4, which regulates *IP3R1* gene expression, required S-palmitoylation for translocation from the cytosol to the nucleus. Remarkably, the S-palmitoylated NFATC4 level in *Cln1*–/– mice was substantially reduced, while that of its phosphorylated inactive form was significantly increased in the cytoplasm. The defect suppressed NFATC4 levels in the nucleus, which reduced *IP3R1* expression. Consequently, reduced lysosomal Ca++ level suppressed catalytic activities of Ca++-dependent lysosomal hydrolases such as cathepsin D and tripeptidyl peptidase-1, which impaired autophagic degradation. Our results reveal a previously unrecognized role of *Cln1*/Ppt1 in lysosomal Ca++ homeostasis and suggest that the defect may contribute to impaired autophagy in INCL.

**Persistent mTORC1 signaling contributes to neuropathology in INCL mice: amelioration by recombinant PPT1 or Akt1 inhibitor.**

Although classically, the lysosome has been considered the terminal organelle for degradation and cellular clearance, emerging evidence indicates that it also relays several nutrient cues to the master growth regulator mTORC1 (mechanistic target of rapamycin complex 1) kinase. Previously, we reported that, in *Cln1*–/– mice, lysosomal acidification is dysregulated owing to mislocalization of a critical subunit (V0a1) of v-ATPase, which
regulates lysosomal acidification. The v-ATPase along with Lamtor1 and SLC38A9 are essential components of the lysosomal nutrient-sensing machinery, which regulates mTORC1 signaling. We found that Lamtor1 and SLC38A9, which are components of the lysosomal nutrient-sensing apparatus, require S-palmitoylation for their localization on the lysosomal membrane and are misrouted in PPT1–deficient Cln1−/− mice. However, despite the disruption of the lysosomal nutrient-sensing machinery, mTORC1 signaling is persistently high in Cln1−/− mouse brains. Notably, in Cln1−/− mice sustained high levels of p-Akt (a serine/threonine-specific protein kinase), p-TSC2 (phospho-tubersclerosis 2), and pPRAS40 (proline-rich Akt substrate of 40 kDa), which promoted the recruitment of Rheb (Ras homolog enriched in brain, a GTP–binding protein) to the lysosomal surface, mediating mTORC1 activation. Remarkably, while mTORC1 signaling in the brain was detectable only on postnatal day 1 of wild-type (WT) mice and was virtually undetectable through adulthood, it was persistently detectable in the brains of Cln1−/− prenatal mice through adulthood, the dysregulated autophagy contributing to neuropathology. Importantly, an Akt1 inhibitor or recombinant PPT1 substantially reduced mTORC1 signaling. Our results reveal a previously unrecognized role of PPT1 in regulating mTORC1 signaling and suggest that inhibitors of Akt1 may have therapeutic implications for INCL.

Publications


Collaborators

- Eva Baker, MD, PhD, *Radiology and Imaging Sciences, Clinical Center, NIH, Bethesda, MD*
- Yichao Li, MS, *Visual Function Core, NEI, Bethesda, MD*
- Chris J. McBain, PhD, *Section on Cellular and Synaptic Physiology, NICHD, Bethesda, MD*
- Kenneth Pelkey, PhD, *Section on Cellular and Synaptic Physiology, NICHD, Bethesda, MD*
- Haohua Qian, PhD, *Visual Function Core, NEI, Bethesda, MD*
- Ling-Gang Wu, PhD, *Synaptic Transmission Section, NINDS, Bethesda, MD*
- Wadih M. Zein, MD, *Ophthalmic Genetics and Visual Function Branch, NEI, Bethesda, MD*

Contact

For more information, email mukherja@exchange.nih.gov or visit https://www.nichd.nih.gov/research/atNICHDIInvestigators/mukherjee.
Gene Regulation in Innate Immunity

The laboratory is interested in chromatin and gene regulation in innate immunity. We study the role of the three nuclear factors histone H3.3, BRD4, and IRF8. Histone H3.3 is a variant H3 that is incorporated into nucleosomes along with transcriptional elongation, an unusual but defining feature of H3.3, given that most of other histones are deposited into nucleosomes during replication. For this reason, H3.3 is thought to be involved in epigenetic memory created by transcription, although experimental evidence for memory formation/maintenance is scant. BRD4 is a bromodomain protein of the BET family, expressed broadly in many cells, from early embryos to adults. Through the bromodomain, BRD4 binds to acetylated histones but not to unacetylated histones. BRD4 is thus called a “chromatin reader,” a type of regulatory factor capable of conveying epigenome information to other regulators of gene expression. Furthermore, BRD4, binds to the elongation factor complex P-TEFb through the C-terminal domain, and drives transcription of many genes by helping RNA polymerase II move through the gene body and to generate nascent mRNA. Many recent reports point out that BRD4 promotes growth of cancer cells, including various blood cancers, by mediating formation of super-enhancers. IRF8, which we reported in 1990 for the first time, is a DNA–binding transcription factor that plays an essential role in innate immune responses. IRF8 is expressed mostly in cells of the myeloid lineage, including monocytes/macrophages, dendritic cells, and microglia. IRF8 is strongly induced when stimulated by interferons (IFN). In addition, it is upregulated when myeloid cells encounter pathogen-derived molecules and those produced by stress. In turn, IRF8 activates many genes important for host resistance against pathogens. IRF8–induced genes include those involved in autophagy and lysosome-mediated pathogen clearance. IRF8 does so by binding to the promoter and enhancer regions of the target genes.

IRF8 shapes adult microglia identity through binding to super-enhancers.

IRF8, along with the transcription factor Spi1 (PU.1), plays a role in early embryonic development of microglia after the progenitors in the yolk sac migrate into the embryonic brain. It is assumed that IRF8 also has a role in adult microglia, given that it is expressed at high levels in such cells. However, the role of IRF8 in adult microglia has not been
FIGURE 1.
IRF8 is highly expressed in adult microglia and confers microglia cell identity. IRF8 KO microglia lack ramified extensions and fail to express MHC II (CD74).

additionally, we found that IRF8 is required to activate two transcription factors critical for adult microglia identity and function, i.e., Sall1 and Batf3.

Furthermore, we determined genome-wide IRF8 distribution in adult microglia using the CUT&RUN technique (which identifies the binding sites of DNA–associated proteins). Our analyses revealed that IRF8 binds mostly over distant enhancer regions, located upstream and downstream of its target genes; there was less IRF8 binding on the immediate promoter regions. Some of IRF8–bound sites were within the large “super-enhancers” enriched with H3K27ac histone marks, which are responsible for strong transcription of genes that create specific cell types. Irf8 KO microglia were devoid of super-enhancers important for shaping microglia identity. In accordance, an ATAC-seq (assay for transposase-accessible chromatin using sequencing) analysis found that IRF8 is important for installing open chromatin necessary for microglia super-enhancers and gene expression. The results imply that IRF8 critically contributes the formation of the microglia-specific epigenome landscape. Further analyses to delineate DNA methylome profiles are ongoing. Our analyses indicate that IRF8 regulates overall CpG–island methylation patterns. In a recently published study on a mouse model of Alzheimer’s disease (AD), it was suggested that IRF8 is involved in the progression of the disease. We investigated an AD mouse model (5FAD) with and without the Irf8 gene. Our transcriptome and other analyses indicate that IRF8 has dual roles and can promote AD pathogenesis in a complex manner.

BRD4 marks cell-cycle genes and orchestrates gene expression necessary for mitotic cell division.
Cell-cycle progression is driven by sequential activation of transcription factors, kinases, and other effectors. BRD4 has been shown to promote the growth of many cancers. Recent publications report that several BRD4 inhibitors (BETi) can arrest cancer growth. BETi thus offer a new possibility for anti-cancer therapy. However, the role of BRD4 in controlling the growth of normal cells has remained elusive. We investigated whether BRD4
FIGURE 2A. BRD4 has two bromodomains and an ET domain. The lab characterized essential features of this factor.

FIGURE 2B. Model for BRD4 regulation of cell cycle progression

Top. In normal cells (WT), BRD4 binds to cell-cycle genes and drives their transcription in a stage-specific manner and orchestrates mitotic cell division.

Bottom. In Brd4 KO cells, cell-cycle genes are not marked by BRD4, and their expression is down-regulated and delayed. Brd4 KO cells are defective in chromatin duplication at S phase and fail to execute mitotic cells division. Many Brd4 KO cells undergo apoptotic cell death, while some KO produce offspring with unequal chromosomal segregation.

also regulates the growth of normal cells by examining embryonic fibroblasts from Brd4 conditional knockout (KO) mice. By conducting growth-curve analysis and an analysis the cell-cycle progression of synchronized cells, we found that BRD4 is critically required for cell-cycle progression of such cells from cell-cycle phases G0/G1, G1 to S and to G2/M. Transcriptome analysis found that genes required for every cell-cycle stage were markedly downregulated in Brd4 KO cells, including several histone genes at S phase, as well as the G2/M master regulators FOXM1 and ATM/ATR. FOXM1 is a transcription factor of the forkhead family and promotes transcription of many G2/M genes. ATM/ATR are kinases previously known to be involved in DNA–damage repair. ATR/ATM were recently shown to play a role in G2/M transition of normal cells. Consistent with these results, BRD4 constitutively occupied numerous cell-cycle genes active at cell-cycle phases G0/G1, S, and G2/M. Interestingly, overall BRD4 occupancy was maximum at S phase. ChIP-seq analysis of BRD4 binding revealed that BRD4 occupies numerous cell-cycle genes at the transcription start site (TSS) and gene body
throughout the entire stages of growth. Thus cell-cycle genes are constitutively marked by BRD, which would provide a platform for continuous cell proliferation. We performed time-lapse imaging analysis to determine the consequence of BRD4 deficiency. We found that the absence of BRD4 culminates in G2/M cells undergoing a catastrophic failure in mitosis. Nuclear materials in Brd4 KO cells disintegrated without assembling at the mitotic plate, leading to apoptosis (Figure 2B). Our study demonstrates that BRD4 orchestrates the entire program of cell-cycle progression for both normal and cancer cells.

**HIRA confers chromatin accessibility on the hematopoietic stem cells and guides entire hematopoietic lineage development.**

We have been interested in the role of histone H3.3, given that it is implicated in transcriptional memory. H3.3 has been shown to localize to actively transcribed and bivalent regions, a process mediated by the H3.3–specific histone chaperone HIRA. H3.3 also localizes to heterochromatin regions including the telomere, where transcription is silenced. The latter process is mediated by another chaperone, ATRX. With the aim of
developing experimental tools useful for clarifying localization and the functional significance, we constructed firstly mouse lines expressing HA–tagged H3.3 and secondly those with conditional H3.3 knockout. Both H3.3 genes (H3f3a and H3f3b) were replaced by H3.3–HA and marked by LoxP (sites that denote nucleotide sequences removable by Cre recombinase). By studying the H3.3–HA mice, we showed that H3.3 is expressed in almost all adult tissues, including immune cells. Genome-wide deposition analysis of embryonic fibroblasts revealed that H3.3–HA is deposited over numerous genes, approximately correlating with levels of mRNA expression.

We are also interested in studying the role of HIRA, because it is involved in genic H3.3 deposition and is likely to control transcription programs and memory formation (Figure 3B). We therefore constructed Hiraf/f mice and generated mice in which the Hira gene is deleted in hematopoietic stem cells (HSCs) by crossing Hiraf/f with the Vav-Cre strain. The mice provide a novel tool with which to elucidate the role of HIRA in hematopoiesis, which is not well understood. The study has some clinical relevance in that patients with DiGeorge syndromes have deletion of an over 11Mb DNA stretch encompassing HIRA. DiGeorge syndromes are little understood diseases, and patients manifest varied abnormalities, including immunodeficiency and thrombocytopenia. Our analysis found that HIRA is essential for the development and the maintenance of bone marrow (BM) HSCs, in that long-term (LT) HSC were almost absent in Hira KO BM. LT-HSCs possess the capacity for self-renewal and progenitor differentiation. Because all adult hematopoietic cells are derived from the HSCs, Hira KO mice were deficient in all three blood lineages, erythroid, myeloid, and lymphoid cells, leading to anemia, thrombocytopenia, and severe immunodeficiency. While some T cells were found in peripheral lymphoid organs, including thymus and lymph nodes, mature B cells were virtually absent from the mice. Such deficiencies were replicated in adoptive transfer experiments, in which wild-type mice reconstituted with Hira KO HSCs failed to generate erythroid, myeloid, and lymphoid lineage cells of Hira KO origin. However, fetal hematopoiesis was normal in Hira KO mice, although fetal HSCs lacked reconstitution capacity. Transcriptome analysis revealed that HIRA is required for expression of many transcription factors and signaling molecules critical for HSCs. ATAC-seq analysis revealed that HIRA establishes HSC–specific DNA accessibility, including the SPI1/PU.1 sites (see Figure 3B for a model). The study demonstrates that HIRA creates a chromatin environment essential for HSCs to acquire self-renewal and differentiation capacity.

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Publications


**Collaborators**
- Steven L. Coon, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Robert J. Crouch, PhD, *Section on Formation of RNA, NICHD, Bethesda, MD*
- Haitong Hou, PhD, *Laboratory of Biochemistry and Molecular Biology, National Cancer Institute, Bethesda MD*
- Yoh-suke Mukouyama, PhD, *Laboratory of Stem Cell and Neuro-Vascular Biology, NHLBI, Bethesda, MD*
- Dinah S. Singer, PhD, *Experimental Immunology Branch, NCI, Bethesda, MD*
- Tomohiko Tamura, MD, PhD, *Tokyo University, Tokyo, Japan*
- Vivek Thumbigere Math, DDS, PhD, *University of Maryland, School of Dentistry, Baltimore, MD*

**Contact**
For more information, email [ozatok@mail.nih.gov](mailto:ozatok@mail.nih.gov) or visit [http://ozatolab.nichd.nih.gov](http://ozatolab.nichd.nih.gov).
Diagnosis, Localization, Pathophysiology, and Molecular Biology of Pheochromocytoma and Paraganglioma

Pheochromocytomas (PHEOs) and paragangliomas (PGLs) are rare but clinically important chromaffin-cell tumors that typically arise, respectively, from the adrenal gland and from extra-adrenal paraganglia. The clinical features and consequences of PHEO/PGL, collectively known as PPGLs, result from the release of catecholamines (norepinephrine and epinephrine). An undetected PHEO/PGL poses a hazard to patients undergoing surgery, childbirth, or general anesthesia because of the potential for excess catecholamine secretion, which can result in significant, often catastrophic outcomes. Diagnosing and localizing a PHEO/PGL can be challenging. Plasma and urinary catecholamines, as well as their metabolites, and radioiodinated metaiodobenzylguanidine (MIBG) scanning can yield false-positive or false-negative results in patients harboring the tumor, and computed tomography (CT) and magnetic resonance imaging (MRI) lack sufficient specificity. The molecular mechanisms by which genotypic changes predispose to the development of PHEO/PGL remain unknown, even in patients with identified mutations. Moreover, in patients with hereditary predispositions, PPGLs differ in terms of their growth, malignant potential, catecholamine phenotype, responses to standard screening tests, various imaging modalities, and therefore to different therapeutic options. We focus on developmental, molecular, genetic, epigenetic, proteomic, metabolomic, immunologic, and other types of studies to investigate the bases for a predisposition to develop PPGLs and the expression of various neurochemical phenotypes and malignant potentials, including therapeutic responses.

Clinical and genetic aspects of pheochromocytoma and paraganglioma

PPGLs are usually benign neuroendocrine tumors. However, PPGLs with mutations in the succinate dehydrogenase B subunit (SDHB) have a poor prognosis and frequently develop metastatic lesions. PPGLs are rare in children, with only a few SDHB mutation–related cases. Previous studies in children were conducted in small cohorts. This large set of pediatric patients provides robust data in the evaluation of clinical outcomes. Thirty-eight males and 26 females were diagnosed with PPGL at a median age of 13 years. The majority of patients displayed norepinephrine hypersecretion, and 73% initially presented...
with a solitary tumor. Metastases developed in 70% of patients at the median age of 16 years and were mostly diagnosed first two-years and then in years 12–18 post-diagnosis. The presence of metastases at the time of diagnosis had a strong negative impact on survival in males but not in females. The estimated 5-, 10-, and 20-year survival rates were 100%, 97%, and 78%, respectively. We highlighted several important aspects in the management of pediatric patients with SDHB mutation–associated PHEO/PGL. Initial diagnostic evaluation of SDHB–mutation carriers should be started at the age of 5–6 years, with initial work-up focusing on the abdominal region. Thorough follow-up is crucial in the first two years post-diagnosis, and more frequent follow-ups are needed in years 10–20 post-diagnosis because of the increased risk of metastases. Although this age group developed metastasis as early as five years from diagnosis, we showed that the overall 20-year prognosis and survival are good.

A PPGL–related clinical sequela results from elevated catecholamine secretion, which can cause hypertension, tachy-arrhythmia, multi-organ failure, and death. We introduced Ivabradine, a commercially available drug that acts directly on the sinus node in the heart for treatment of severe catecholamine-induced tachy-arrhythmia. We also published some comprehensive reviews on cardiac PGLs, as well as on treatments of arrhythmias.

In another study, we evaluated PPGL patients with the SDHA gene mutation. Our findings suggest that such tumors can occur early and at extra-adrenal locations, behave aggressively, and have a tendency to develop metastatic disease within a short period of time. None of the patients had a family history of PPGL, making them appear sporadic. Nine out of 10 patients showed abnormal PPGL–specific biochemical markers with predominantly noradrenergic and/or dopaminergic phenotype, suggesting their utility in diagnosing and monitoring the disease. A radioconjugate, $^{68}$Ga-DOTATATE PET, consisting of the somatostatin analog tyrosine-3-octreotate (Tyr3-octreotate or TATE) labeled with the positron emission tomography (PET) tracer gallium Ga 68 via the macroyclic chelating agent dodecanetetraacetic acid (DOTA), which may be used as a somatostatin receptor imaging agent in conjunction with PET, was superior to other imaging modalities in localizing these tumors. All seven patients who received conventional therapies (chemotherapy, somatostatin-analog therapy, radiation therapy, $^{131}$I-MIBG, peptide-receptor radionuclide therapy) in addition to surgery showed progression.

Brown adipose tissue (BAT) activation is mediated through the action of norepinephrine on β-adrenoceptors (β-ARs). In some malignancies, BAT activation is associated with higher cancer activity. A retrospective case-control study included 342 patients with PPGLs who underwent $^{18}$F-fluoro-2-deoxy-D-glucose PET–computed tomography ($^{18}$F-FDG PET/CT) imaging at the National Institutes of Health (NIH). The presence of active BAT on $^{18}$F-FDG PET/CT was associated with lower overall survival than in the control group. The association remained significant after adjusting for the SDHB mutation. Median plasma norepinephrine in the BAT group was higher than the control group. There was a significant association between higher plasma norepinephrine levels and mortality in PPGLs in both groups.

**Imaging of pheochromocytomas and paragangliomas**

Diverse radionuclide imaging techniques are available for the diagnosis, staging, and follow-up of PPGL. Beyond their ability to detect and localize the disease, the imaging approaches variably characterize the tumors at the cellular and molecular levels and can guide therapy. We updated guidelines jointly approved by the EANM (European Association of Nuclear Medicine) and SNMMI (Society of Nuclear Medicine and Molecular Imaging) for assisting nuclear-medicine practitioners in not only the selection and performance of currently available single-photon emission computed tomography and PET procedures, but also the

The initial phase of immunotherapy based on the combination of TLR ligands, mannan–BAM, and anti–CD40 antibody.

A. After intra-tumoral application of the therapy (TLR ligands, mannan–BAM, anti–CD40 antibody), tumor cells are artificially opsonized by mannan–BAM, where the terminal part of BAM is incorporated into the lipid bilayer of tumor cells.

B. Mannan–BAM is subsequently recognized by mannan-binding lectin (MBL). The recognition results in activation of the complement system followed by proteolytic cleavage of complement protein C3 and the production of the terminal membrane attack complex (MAC). During the proteolytic cleavage of C3 into C3a and C3b, the inactive form of C3b (iC3b) opsonizes the tumor cells. Simultaneously, the TLR ligands R-848, poly(I:C), and LTA are recruiting immune cells into the tumor.

C. Tumor cells opsonized by iC3b are recognized by innate immune cells (NK cells, neutrophils, macrophages) previously recruited into the tumor. Such innate immune cells use their effector mechanisms to kill the opsonized tumor cells. As a part of the therapy, anti–CD40 antibodies bind to CD40 receptors expressed mainly on antigen-presenting cells (macrophages, dendritic cells) and initiate their activation. The activated antigen-presenting cells further internalize tumor antigens and present them to T cells in lymph nodes.

interpretation and reporting of the results from PPGL patients. We also recently published a review about molecular imaging and radionuclide therapy of PPGL in the era of genomic characterization.

Immune and metabolic aspects of pheochromocytoma and paraganglioma

Therapeutic options for metastatic PHEO/PGL are limited. We therefore tested an immuno-therapeutic approach based on intra-tumoral injections of the antibiotic complex mannan–BAM (biocompatible anchor for membranes) with toll-like receptor ligands (TLRs) into subcutaneous PHEOs in a mouse model (Figure 1). The therapy elicited a strong innate immunity-mediated antitumor response and resulted in a significantly lower PHEO volume compared with the phosphate buffered saline (PBS)–treated group and in a significant improvement in mouse survival. We verified the cytotoxic effect of neutrophils, as innate immune cells predominantly infiltrating treated tumors, in vitro. Moreover, the combination of mannan–BAM and TLRs with agonistic anti–CD40 (CD40 receptors are expressed mainly on antigen-presenting cells such as macrophages and dendritic cells) was associated with increased mouse survival. Subsequent tumor re-challenge also supported adaptive immunity activation, reflected primarily by long-term tumor-specific memory. We verified
these results further in metastatic PHEO, where the intra-tumoral injections of mannan–BAM, TLRs, and anti–CD40 into subcutaneous tumors resulted in significantly less intense bioluminescence signals of liver metastatic lesions induced by tail vein injection than in the PBS–treated group. Subsequent experiments focusing on the depletion of T cell subpopulations confirmed the crucial role of CD8+ T cells in the inhibition of bioluminescence signal intensity of liver metastatic lesions. The results call for a new therapeutic approach in patients with metastatic PHEO/PGL by using immunotherapy that initially activates innate immunity, followed by an adaptive immune response.

**Therapeutic aspects of pheochromocytoma and paraganglioma**

SDHB–mutated PPGLs exhibit dysregulation in oxygen-metabolic pathways, including pseudohypoxia and the formation of reactive oxygen species, suggesting that targeting the redox balance pathway is a potential therapeutic approach. By investigating PPGL cells with low SDHB levels, we showed that pseudohypoxia resulted in elevated expression of iron-transport proteins, including transferrin (TF), transferrin receptor 2 (TFR2), and the divalent metal transporter 1 (SLC11A2; DMT1), leading to iron accumulation. The iron overload contributed to elevated oxidative stress. At pharmacologic concentrations, ascorbic acid disrupted redox homeostasis, inducing DNA oxidative damage and cell apoptosis in PPGL cells with low SDHB levels. Moreover, using a preclinical animal model with PPGL allografts, we demonstrated that pharmacologic ascorbic acid suppressed SDHB–low metastatic lesions and prolonged overall survival. The data demonstrate that targeting redox homeostasis as a cancer vulnerability with pharmacologic ascorbic acid is a promising therapeutic strategy for SDHB–mutated PPGLs.

Mechanistically, nuclear factor erythroid 2–related factor 2 (NRF2)–guided glutathione de novo synthesis plays a key role in supporting cellular survival and the proliferation of SDHB–knockdown cells. We found that NRF2 blockade not only disrupted reactive oxygen species homeostasis in SDHB–deficient cells but also caused severe cytotoxicity by the accumulation of DNA oxidative damage. Brusatol, a potent NRF2 inhibitor, showed a promising effect in suppressing SDHB–gene metastatic lesions in vivo, with prolonged overall survival in mice bearing PPGL allografts. Our findings highlight a novel therapeutic strategy of targeting the NRF2–driven glutathione metabolic pathway against SDHB–mutated PPGLs.

PPGLs, arising from chromaffin cells, produce the catecholamines epinephrine and norepinephrine. The tumor biochemical phenotype is defined by which of these exerts the greatest influence on the cardiovascular system when released into circulation in high amounts. Action on the heart and vasculature can cause potentially lethal arrhythmias, often in the setting of comorbid blood pressure derangements. In a review of electrocardiograms obtained from PPGL patients (n = 650) treated at our institution over the last decade, we found severe and refractory sinus tachycardia, atrial fibrillation, and ventricular tachycardia to be the most common or life-threatening catecholamine-induced tachy-arrhythmias. These arrhythmias, arising from catecholamine excess rather than from a primary electro-physiologic substrate, require special considerations for treatment and complication avoidance. Understanding the synthesis and release of catecholamines, the adrenoceptors that catecholamines bind to, and the cardiac and vascular response to epinephrine and norepinephrine underlies optimal management in catecholamine-induced tachy-arrhythmias. Therefore, in a recent review we outlined tachy-arrhythmias in PPGLs and their treatment options.

As the member of the Working group on Endocrine Hypertension of the European Society of Hypertension, we outlined the newest approaches to the evaluation and treatment of a patient with PPGLs based on current
knowledge in PPGL epidemiology, genetics, diagnosis, and treatment. We also wrote a review as a guide for practicing clinicians summarizing current management of PHEO/PGL according to tumor size, location, age of first diagnosis, presence of metastases, and especially underlying mutations, in the era of precision medicine.

Animal model of pheochromocytoma and cell culture studies
We previously identified the syndrome of multiple paragangliomas and pheochromocytomas, duodenal somatostatinoma, and polycythemia resulting from post-zygotic EPAS1 (HIF2A)-gain-of-function mutations (also called Pacak-Zhuang syndrome). The mutations, located in the oxygen-degradation domain (ODD) of hypoxia-inducible factor-2a (HIF-2a), have been shown to impair hydroxylation by prolyl hydroxylase domain-containing protein 2 (PHD2) and subsequent association with the von Hippel-Lindau (VHL) protein. In such a situation, degradation of HIF-2a is impaired, resulting in its stabilization, prolonged activation, lack of response to normal or increasing oxygen tension, and activation of the transcription of many genes participating in tumorigenesis. Recently, together with the collaboration of NCI investigators, we developed transgenic mice with a gain-of-function Epas1A529V mutation (corresponding to human Epas1A530V), which demonstrated elevated levels of erythropoietin and polycythemia, a decreased urinary metanephrine-to-normetanephrine ratio, and increased expression of somatostatin in the ampullary region of duodenum. The findings demonstrate the vital roles of EPAS1 mutations in the syndrome development and the great potential of the Epas1A529V animal model for further pathogenesis and therapeutics studies. The model is also being used to study other malformations in animals as well as to match them with those seen in our patients (neurological, vascular, and ocular malformations).

Publications

Collaborators
- Zahraa Abdul Sater, MD, MPH, Clinical Endocrine Section, NIDDK, Bethesda, MD
- James Bibb, PhD, University of Alabama Comprehensive Cancer Center, University of Alabama at Birmingham Medical Center, Birmingham, AL
- Clara C. Chen, MD, Nuclear Medicine Department, Clinical Center, NIH, Bethesda, MD
- Marilo Chiara, PhD, Hospital Universitario Central de Asturias, Oviedo, Spain
Contact
For more information, email karel@mail.nih.gov or visit http://pheopara.nichd.nih.gov.
The incredible diversity and heterogeneity of interneurons was observed over a century ago, with Ramon y Cajal hypothesizing in "Recollections of My Life" that “The functional superiority of the human brain is intimately linked up with the prodigious abundance and unaccustomed wealth of the so-called neurons with short axons.” Although interneurons constitute the minority (20%) of neurons in the brain, they are the primary source of inhibition and are critical components in the modulation and refinement of the flow of information throughout the nervous system. Abnormal development and function of interneurons has been linked to the pathobiology of numerous brain diseases, such as epilepsy, schizophrenia, and autism. Interneurons are an extremely heterogeneous cell population, with distinct morphologies, connectivities, neurochemical markers, and electrophysiological properties. With the advent of new technologies such as single-cell sequencing to dissect gene expression and connectivity patterns, the classification of interneurons into specific subtypes is ever evolving.

Interneurons such as GABAergic projection neurons are born in the ventral forebrain during embryogenesis and undergo a prolonged migratory period to populate nearly every brain region. However, our general understanding of the developmental mechanisms that generate such GABAergic cell diversity remains poorly understood. The goal of our lab is to dissect the genetic and molecular programs that underlie initial fate decisions during embryogenesis and to explore how the environment and genetic cascades interact to give rise to such a stunning diversity of GABAergic cell subtypes. We take a multifaceted approach, utilizing both in vitro and in vivo strategies to identify candidate mechanisms that regulate interneuron fate decisions. We strive to develop cutting-edge techniques that will overcome the many challenges faced when studying interneuron development. We believe that our pursuits will act as a springboard for future research and provide new insights into both normal development and various neurodevelopmental diseases.

Mechanisms regulating initial fate decisions within the medial ganglionic eminence
The medial ganglionic eminence (MGE) gives rise to the majority
of forebrain interneurons, most notably the somatostatin- and parvalbumin-expressing (SST+ and PV+) subtypes, and some nNOS (neuronal nitric oxide synthetase)-expressing neurogliaform and ivy cells in the hippocampus. The MGE is a transient, dynamic structure that arises around E10 and bulges into the lateral ventricle over the next several days before dissipating towards the end of embryogenesis. Given that initial fate decisions are generated within the MGE, there has been much focus on identifying a logic for interneuron generation from this region. Previous experiments characterized both a spatial and temporal gradient within the MGE that regulates the initial fate decision to become either PV+ or SST+ interneurons. SST+ interneurons

FIGURE 1. MGE–derived GABAergic cells populate many different brain regions.

The image depicts a section of an embryonic brain (left) that has been electroplated to label cells derived from the medial ganglionic eminence (MGE), merged with an section of an adult brain (right), displaying the incredible spatial and morphological diversity of MGE–derived cells in the mature brain. Understanding how this heterogeneous population is generated from one embryonic brain structure is the focus of this laboratory.

FIGURE 2. Manipulation of gene expression in the MGE by in utero electroporation (IUE)

Top. By using Nkx2.1-Cre mice, we can restrict expression of cre-dependent plasmids to the MGE. Note that the GFP+ cells are restricted to the MGE and to cells migrating away from the MGE at 2 days post-electroporation.

Bottom. Representative example from a P25 cortex of a mouse whose MGE was electroplated at E12.5. In this series of experiments, we used IUE to demonstrate that the mode of neurogenesis plays a role in interneuron fate determination: apical neurogenic divisions are biased to generate somatostatin (SST+) interneurons, whereas basal divisions primarily give rise to parvalbumin (PV+) cells.
are preferentially born early in embryogenesis from the dorsoposterior MGE, whereas PV⁺ interneurons are born throughout embryogenesis with a bias of originating from the ventroanterior MGE. We discovered an additional mechanism regulating this fate decision: the mode of neurogenesis. Using *in utero* electroporations, we found that PV⁺ interneurons are preferentially born from basal progenitors (also known as intermediate progenitors), whereas SST⁺ interneurons arise more commonly from apical progenitors. We hope to build on this observation to discover how these distinct spatial, temporal, and neurogenic gradients coordinate to regulate initial fate decisions of MGE progenitors.

**Characterization of the epigenetic landscape during embryonic neurogenesis**

In multicellular organisms, cells are genetically homogenous but structurally and functionally heterogeneous as a result of differential gene expression, which is often mitotically heritable. The mechanisms regulating such expression are ‘epigenetic,’ as they do not involve altering the DNA sequence itself; they include DNA methylation (DNAme), histone modifications, and higher-order chromatin structure. In particular, DNA and histone modifications often follow specific rules termed the “epigenetic code,” similar to the genetic code. Collectively, DNAme and histone modification have been reported to regulate transcription and chromatin structure in many stem-cell and developmentally critical processes. Previous scRNA-Seq (single-cell RNA sequencing) experiments on the ganglionic eminences (GEs) identified surprisingly few region-specific genes in cycling progenitors (immature cells that are still cycling and have not exited the cell cycle), despite the fact that these regions produce distinct GABAergic cell populations. Because there are dynamic changes in the chromatin landscape during development, a prevailing hypothesis is that epigenomic signatures may be a better predictor of cell fate during development, revealing both potential distal enhancers and/or genetic loci that may be ‘poised’ but not yet expressed. However, direct support for this hypothesis is lacking. The idea is particularly relevant, given that epigenetic changes are observed in many neurological and psychiatric diseases and that most single-nucleotide variants (SNVs) identified in diseases-specific GWAS (genome-wide association studies) map to non-coding regions, implying that epigenetic regulation of gene expression may underlie some disease etiologies. We are currently using cutting-edge techniques to define the chromatin state of different embryonic neurogenic regions to better understand epigenetic changes in distinct cell types during development, with the hope applying this knowledge to various neuro-developmental diseases.
How the environment sculpts interneuron diversity and maturation.

Interneurons undergo an extensive tangential migration period before reaching their terminal brain region, whereupon they interact with the local environment to differentiate and mature. The composition of interneuron subtypes varies significantly between different brain regions. Numerous experiments indicate that general interneuron classes, e.g., PV+– or SST+–expressing interneurons, are determined as cells become post-mitotic during embryogenesis. However, when other features that define a mature interneuron subtype (neurochemical markers, cell type and subcellular location of synaptic partners, electrophysiology properties, etc.) are established remains unknown. One hypothesis is that interneurons undergo an initial differentiation into ‘cardinal’ classes during embryogenesis, and that maturation into ‘definitive’ subgroups requires active interaction with their mature environment. An alternative hypothesis is that immature interneurons are already genetically hardwired into definitive subgroups, and that the environment more passively sculpts the maturation of these cells. To test the competing hypotheses, we are harvesting early postnatal interneuron precursors (P0–P2) in specific brain regions and transplanting them into wild-type (WT) mice. The technique allows us to determine whether transplanted interneurons adopt properties of the host environment (indicating a strong role for the environment in regulating interneuron diversity) or retain subtype features more consistent with the donor region. Our initial experiments indicate that the environment largely determines the composition of interneuron subtypes in a brain region, regardless of donor region. However, some interneuron subtypes appear to be more genetically predefined and resistant to environmental influences than others. We are currently following up on these studies using scRNA-Seq to characterize, in an unbiased manner, how a cell's transcriptome is altered when grafted into a new brain environment.

Novel approach to identifying genetic cascades underlying interneuron fate decisions

The ability to longitudinally track gene expression within defined populations is essential for understanding how changes in expression mediate both development and plasticity. Previous screens that were designed to
FIGURE 5. Timestamp of actively transcribed genes during development for future analysis

The goal of this approach is to label actively transcribed genes with stable methylation tags during embryogenesis as progenitors are undergoing initial fate decisions in the MGE. We can then harvest specific interneuron subtypes in the adult brain using various transgenic mouse lines. Retrospective identification of an actively transcribed gene during embryogenesis will provide us with candidate fate-determining genes for specific interneuron subtypes.

identify genes and transcription factors specific to SST- or PV-fated interneurons were largely unsuccessful because several issues significantly hinder these types of studies. First, these interneurons originate from the MGE, which is a heterogeneous population of progenitors that give rise to both interneurons and a variety of GABAergic projection neurons, making it difficult to segregate interneuron progenitors from other cell types. Additionally, many markers that define mature interneuron subtypes are not expressed embryonically, and thus the class-defining markers are not helpful for studying MGE progenitors. In an ideal scenario, we would like to identify actively transcribed genes in MGE progenitors undergoing fate decisions while retaining the capacity to identify whether these cells become PV- or SST-expressing interneurons in the postnatal brain. To this end, we are developing a spatially and temporally inducible form of DNA adenine methylase identification (DamID), which will allow us to label the transcriptome of MGE progenitors. Labeled cells can be harvested at maturity, once we have the tools to distinguish between specific interneuron cell types. Then, the methylated genomic DNA will be analyzed, allowing us to look back in time to identify candidate fate-determining genes expressed in specific interneuron populations. Our hope is that the strategy could be widely applicable so that an investigator could characterize the temporal gene expression pattern of the cell type of interest.

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Publications

Collaborators

• Susan Amara, PhD, Laboratory of Molecular and Cellular Neurobiology, NIMH, Bethesda, MD
• Andrea Brand, PhD, The Gurdon Institute, University of Cambridge, United Kingdom
• Ryan Dale, PhD, Bioinformatics Core, NICHD, Bethesda, MD
• Claire Le Pichon, PhD, Unit on the Development of Neurodegeneration, NICHD, Bethesda, MD
• Chris McBain, PhD, Section on Cellular and Synaptic Physiology, NICHD, Bethesda, MD
• Isabel Perez-Otano, PhD, Instituto de Neurociencias de Alicante, Alicante, Spain

Contact

For more information, email tim.petros@nih.gov or visit https://www.nichd.nih.gov/research/atNICHD/Investigators/petros.
Molecular Genetics of an Imprinted Gene Cluster on Mouse Distal Chromosome 7

Genomic imprinting is an unusual form of gene regulation by which an allele’s parental origin restricts allele expression. For example, almost all expression of the noncoding RNA tumor-suppressor gene H19 is from the maternal chromosome. In contrast, expression of the neighboring Insulin-like Growth Factor 2 gene (Igf2) is from the paternal chromosome. Imprinted genes are not randomly scattered throughout the chromosome but rather are localized in discrete clusters, where monoallelic expression is regulated by a common cis-acting DNA regulatory element called the Imprinting Control Region (ICR). We study a cluster of imprinted genes on the distal end of mouse chromosome 7 (Figure 1). The syntenic region in humans (11p15.5) is highly conserved in gene organization and expression patterns. Imprinting of H19 and of Igf2 is regulated by the H19ICR, which is located just upstream of the H19 promoter. We showed that the molecular function of the H19ICR is to organize the region into alternative 3D structures. In humans, epigenetic mutations that disrupt H19ICR function result in loss of monoallelic expression. Mutations in the paternal H19ICR lead to loss of Igf2 expression and biallelic (2X) H19 expression and are associated with the Russell-Silver syndrome. Mutations in the maternal H19ICR lead to loss of Igf2 but biallelic (2x) H19 expression and are associated with the Beckwith-Wiedemann syndrome and several pediatric cancers. Our lab generated mouse models that phenocopy the human diseases, and our goal is to characterize the molecular defects associated with mis-expression of Igf2/H19 and to understand how these molecular defects lead to disease and cancer. In particular, we strive to understand the role of development in disease progression. In addition to the Igf2/H19 locus, we are completing analyses of mice carrying conditional mutations at the Casq2 locus to understand the role of development in cardiac disease progression.

Alternative long-range interactions between distal regulatory elements establish allele-specific expression at the Igf2/H19 locus.

Paternally expressed Igf2 lies about 80 kb upstream of the maternal-specific H19 gene. Using cell-culture systems as well as transgene and knockout experiments in vivo, we identified the enhancer elements responsible for activation of the two genes. The elements...
are shared and are all located downstream of the \textit{H19} gene (Figure 2). As mentioned above, imprinting at the \textit{Igf2}/\textit{H19} locus depends on the 2.4 kb \textit{H19ICR}, which lies between the two genes, just upstream of the \textit{H19} promoter (Figure 2). On the maternal chromosome, binding of the CTCF protein, a transcriptional repressor, to the \textit{H19ICR} establishes a transcriptional insulator that organizes the chromosome into loop structures that bring the \textit{H19} promoter into contact with downstream enhancers but exclude the \textit{Igf2} promoter from these enhancer interactions. The loops favor \textit{H19} expression but block interactions between the maternal \textit{Igf2} promoters and the downstream shared enhancers, thus preventing maternal \textit{Igf2} expression. Upon paternal inheritance, the cytosine residues within the ICR DNA sequences are methylated, which prevents binding of the CTCF protein, so that a transcriptional insulator is not established. Thus, paternal \textit{Igf2} promoters and the shared enhancers interact via DNA loops, and expression of paternal \textit{Igf2} is facilitated. Taken together, we find that the fundamental role of the ICR is to organize the chromosomes into alternative 3-D configurations that promote or prevent expression of the \textit{Igf2} and \textit{H19} genes. The \textit{H19} ICR is not only necessary but is also sufficient for genomic imprinting. To demonstrate this, we used knock-in experiments to insert the 2.4 kb element at heterologous loci and demonstrated its ability to imprint these regions. Further, analyses of the loci confirmed and extended the transcriptional model described above. Upon maternal inheritance, even ectopic \textit{ICR} elements remain unmethylated, bind to the CTCF protein, and form transcriptional insulators. Paternally inherited ectopic ICRs become methylated, cannot bind to CTCF, and therefore promote alternative loop domains distinct from those organized on maternal chromosomes. Most curious was the finding that DNA methylation of ectopic ICRs is not acquired until relatively late in development, after the embryo implants in the uterus. In contrast, at the endogenous locus, ICR methylation occurs during spermatogenesis.
The findings thus imply that DNA methylation is not the primary imprinting mark that distinguishes maternally from paternally inherited ICRs.

The Nctc1 gene lies downstream of H19 and encodes a spliced, polyadenylated long noncoding RNA (lncRNA) that is transcribed across the muscle enhancer element (ME in Figure 2), which is shared by Igf2 and H19. Nctc1 expression depends on this enhancer element. Concordantly, the shared enhancer interacts with the Nctc1 promoter, just as it interacts with the maternal H19 and paternal Igf2 promoters. We showed that all three co-regulated promoters (Igf2, H19, and Nctc1) also physically interact with each other in a manner that depends on their interactions with the shared enhancer. Thus, enhancer interactions with one promoter do not preclude interactions with another promoter. Moreover, we demonstrated that such promoter-promoter interactions are regulatory; they explain the developmentally regulated imprinting of Nctc1 transcription. Taken together, our results demonstrate the importance of long-range enhancer-promoter and promoter-promoter interactions in physically organizing the genome and establishing the gene expression patterns that are crucial for normal mammalian development.

**Molecular mechanisms for tissue-specific promoter activation by distal enhancers**

Normal mammalian development is absolutely dependent on establishing the appropriate patterns of expression of thousands of developmentally regulated genes. Most often, development-specific expression depends on promoter activation by distal enhancer elements. The Igf2/H19 locus is a highly useful model system for investigating mechanisms of enhancer activation. First, the biological significance of the model is clear, given that expression of these genes is so strictly regulated. Even twofold changes in RNA levels are associated with cancer and developmental disorders. Second, we already know much about the enhancers in this region and have established powerful genetic tools to investigate their function. Igf2 and H19 are co-expressed throughout embryonic development and depend on a series of tissue-specific enhancers that lie between 8 and more than 150 kb downstream of the H19 promoter (or between 88 and more than 130 kb downstream of the Igf2 promoters). The endodermal and muscle enhancers have been precisely defined, and we generated mouse strains carrying deletions that completely abrogate enhancer function. We also generated insulator insertion mutations that specifically block muscle enhancer activity. We used these strains to generate primary myoblast cell lines so that we can combine genetic, molecular, biochemical, and genomic analyses to understand the molecular bases for enhancer functions.
**THE LNCRNA ENCODED BY NCTC1 IS AN ESSENTIAL ELEMENT OF THE MUSCLE ENHANCER.**

Transient transfection analyses define a 300-bp element that is both necessary and sufficient for maximal enhancer activity. However, stable transfection and mouse mutations indicate that this core element is not sufficient for enhancer function in a chromosomal context. Instead, the Nctc1 promoter element is also essential; the Nctc1 RNA itself is not required (at least in trans). Instead mutational analysis demonstrates that it is Nctc1 transcription through the core enhancer that is necessary for enhancer function. Curiously, the Nctc1 promoter has chromatin features typical of both a classic enhancer and a classic peptide-encoding promoter. Several recent genomic studies also suggested a role for noncoding RNAs in gene regulation and enhancer function. We will use our model system to characterize the role of Nctc1 transcription in establishing enhancer orientation, enhancer promoter specificity, and enhancer tissue specificity.

**THE MUSCLE ENHANCER (ME) DIRECTS RNA POLYMERASE (RNAP) II NOT ONLY TO ITS COGNATE PROMOTERS (I.E., TO THE H19 AND IGF2 PROMOTERS) BUT ALSO ACROSS THE ENTIRE INTERGENIC REGION.**

We used ChIP-on-chip to analyze RNAP localization on chromatin prepared from wild-type and from enhancer-deletion (DME) cell lines (Figure 3). As expected, RNAP binding to the H19 and Igf2 promoters is entirely enhancer-dependent. Curiously, we also noted enhancer-dependent RNAP localization across the entire locus, including the large intergenic domain between the two genes. Furthermore, RNAP binding is associated with RNA transcription. Thus, the enhancer regulates accessibility and RNAP binding not only at specific localized sites but across the entire domain. The results support a facilitated tracking model for enhancer activity.

**RNAP BINDING AT ‘REAL’ GENES AND ACROSS THE INTERGENIC REGIONS IS QUALITATIVELY DIFFERENT.**

We used naturally occurring single-nucleotide polymorphisms (SNPs) to investigate allelic differences in binding of RNAP and activation of gene expression in wild-type cells and in cells carrying enhancer deletions or insulator insertion mutations. RNAP binding across the Igf2 and H19 genes is both enhancer-dependent and insulator-sensitive; that is, a functional insulator located between an enhancer and its regulated gene prevents RNAP binding and likewise prevents RNA transcription. Across the intergenic regions, RNAP binding and RNA transcription are similarly enhancer-dependent (see above). However, intergenic RNAP binding and transcription are not insulator-sensitive. The results indicate that insulators do not serve solely as a physical block for RNAP progression, but rather they specifically interfere with certain RNAP states or activities.
Figure 5. Cardiac disease in H19-deficient mice

Mice lacking H19 are hypertrophic, fibrotic (panel A), display protein expression profiles typical of cardiac failure (panel B), and show aberrant function on echocardiograms.

The muscle enhancer regulates RNAP binding and RNA transcription, but does not establish chromatin structures.

Both RNA transcription and RNAP binding across the Igf2/H19 domain are entirely dependent upon the muscle enhancer. For example, levels of H19 RNA are reduced more than 10,000-fold in muscle cells in which the enhancer has been deleted. To test the dependence of chromatin structure on enhancer activity, we performed ChiP-seq on wild-type and on enhancer-deletion cell lines using antibodies to the histones H3K4me1, H3K43me3, and H3K36me3. Surprisingly, we saw no changes in the patterns of chromatin modification (Figure 4). Thus, a functional enhancer and active RNA transcription are not important for establishing chromatin structures at the Igf2/H19 domain.

Functions of H19 IncRNA in regulating cell-cycle progression and senescence

To determine the biochemical functions for H19 IncRNA, we are using in vitro models including primary myoblasts, C2C12 myoblasts, and NIH3T3 cells. Abrupt depletion of H19 by either siRNA or by cre-induced recombination of H19-floxed alleles results in increased p21 RNA (p21 is a cyclin-dependent kinase inhibitor involved in cell-cycle arrest) and peptide, and such increased p21 activity in turn prevents cell-cycle progression and induces cellular senescence. H19 IncRNA interacts physically with p21 mRNA and alters p21 mRNA stability and translation. Current experiments focus on identifying the molecular mechanisms for these regulatory actions.

Functions of H19 IncRNA in regulating cardiac development

Beckwith-Wiedeman syndrome (BWS) is a developmental disorder characterized by generalized overgrowth of the fetus and a high risk for several neonatal cancers. Many BWS patients also display cardiac problems. BWS can be explained by one of two different genetic lesions: loss of function of the CDKN1C gene or maternal loss of imprinting at the H19/Igf2 locus. Maternal loss of imprinting has the effect of doubling Igf2 expression while concomitantly reducing H19 RNA levels. Curiously, children born via artificial reproductive technology (ART) show increased incidence of BWS, which can be explained by increasingly frequent loss of H19/Igf2 imprinting in these children. Moreover the children show high frequency of cardiac dysfunction. Altogether, these results suggest that abnormal expression of the H19/Igf2 locus can lead to cardiac problems.
We observed that our BWS mouse model also results in cardiac dysfunction, as measured by echocardiography and ECG analyses. Molecular and molecular-genetic analyses demonstrate that biallelic Igf2 and loss of H19 play independent and distinct roles in generating the BWS phenotype. Biallelic expression of Igf2 results in increased levels of circulating IGF2 peptide, which super-activates insulin and insulin-like receptor kinases in cardiomyocytes, resulting in hyper-activation of AKT/mTOR signaling pathways, which in turn causes cardiomyocyte hypertrophy and hyperplasia. Such effects result in a cardiac hypertrophy that is non-pathologic and transient, i.e., the hearts function normally and, as long as H19 levels are normal, the heart size normalizes after birth once Igf2 expression is repressed. Thus, there are no significant health effects associated with loss of imprinting of Igf2 only.

Loss of expression of H19 is pathologic (Figure 4). Hearts show progressive heart disease as manifested by hypertrophy, increased fibrosis, expression of cardiac failure markers, and reduced and abnormal heart function, as measured by echocardiography. H19 expression in hearts is restricted to endothelial cells. In vivo analyses of whole hearts and in vitro analyses of isolated endothelial cells show that reduction in H19 results in increased endothelial-to-mesenchymal transition (EMT). EMT is an essential feature of normal cardiac development; for example, formation of cardiac valves requires EMT. However, elevated frequency of EMT is associated with heart disease. Our data support the idea that H19 regulates the cell fate of endothelial cells, and future experiments aim to identify the molecular mechanisms.

Role of calsequestrin2 in regulating cardiac function

Mutations in the CASQ2 gene, which encodes cardiac calsequestrin (CASQ2), are associated with catecholaminergic polymorphic ventricular tachycardia (CPVT) and sudden death. The survival of individuals homozygous for loss-of-function mutations in CASQ2 was surprising, given the central role of Ca^{2+} ions in excitation-contraction (EC) coupling and the presumed critical roles of CASQ2 in regulating Ca^{2+} release from the sarcoplasmic reticulum (SR) into the cytoplasm. To address this paradox, we generated a mouse model for loss of Casq2 gene activity. Comprehensive analysis of cardiac function and structure yielded several important insights into CASQ2 function. First, CASQ2 is not essential to provide sufficient Ca^{2+} storage in the SR of the cardiomyocyte. Rather, a compensatory increase in SR volume and surface area in mutant mice appears to maintain normal Ca^{2+} storage capacity. Second, CASQ2 is not required for the rapid, triggered release of Ca^{2+} from the SR during cardiomyocyte contraction. Rather, the RyR receptor, an intracellular calcium-ion channel, opens appropriately, resulting in normal, rapid flow of Ca^{2+} into the cytoplasm, thus allowing normal contraction of the cardiomyocyte. Third, CASQ2 is required for normal function of the RyR during cardiomyocyte relaxation. In the absence of CASQ2, significant Ca^{2+} leaks occur through the RyR and lead to premature contractions and cardiac arrhythmias (Figure 6). Fourth, CASQ2 function is required to maintain normal levels of the SR proteins junctin and triadin. We do not yet understand what role, if any, the compensatory changes in these two SR proteins play in modulating the loss of Casq2 phenotype.

To address these issues and to model cardiac disorders associated with late-onset (not congenital) loss of CASQ2 activity, we established and are now analyzing two new mouse models in which changes in Casq2 gene structure are induced by tissue-specific transgenes activated by tamoxifen treatment. In the first model, an invested/null allele is restored to normal function by the addition of the drug. In the past year, we demonstrated the effectiveness of this model and noted that full CASQ2 protein levels are restored within one week of treatment. In the second model, a functional gene is ablated by the addition of the drug. The Casq2 gene and mRNAs are deleted from cardiac cells within four days of hormone treatment. Phenotypic
analyses show that restoration of CASQ2 in adult animals is sufficient to fully restore cardiac function. Moreover, restoration solely in pacemaking cells is also enough to rescue function, suggesting an important role for reduced heart rate in the CPVT phenotype as well as a new target for therapeutic interventions.

We are also completing experiments to understand the role of normal aging in the CASQ2–dependent arrhythmias. Young mice (less than 6 weeks of age) are entirely asymptomatic even though the cardiomyocytes themselves show the same molecular and electrophysiology defects as those seen in older animals. Instead, genomic analysis indicates that the more severe phenotypes in older mice are associated with global changes in RNA transcription, especially in genes related to energy metabolism.

**Publications**


**FIGURE 6. Cardiac arrhythmias in calsequestrin-2–deficient mice phenocopy the human disease.**

Premature ventricular complexes (*) are induced by stress in Casq2–deficient but not in wild-type mice.
Collaborators

- Judy Kassis, PhD, Section on Gene Expression, NICHD, Bethesda, MD
- Beth Kozel, MD, PhD, Cardiovascular & Pulmonary Branch, NHLBI, Bethesda, MD
- Danielle A. Springer, VMD, Dipl ACLAM, Animal Program, NHLBI, Bethesda, MD

Contact

For more information, email kpfeifer@helix.nih.gov or visit http://pfeiferlab.nichd.nih.gov.
We study the molecular, biochemical, and cellular processes that underlie genetic disorders resulting from impaired cholesterol homeostasis and lysosomal dysfunction. The disorders include malformation/cognitive impairment syndromes resulting from inborn errors of cholesterol synthesis and neurodegenerative disorders resulting from impaired intracellular cholesterol and lipid transport. Human malformation syndromes attributable to inborn errors of cholesterol synthesis include Smith-Lemli-Opitz syndrome (SLOS), lathosterolosis, desmosterolosis, X-linked dominant chondrodysplasia punctata type 2 (CDPX2), and the CHILD syndrome. Niemann-Pick disease type C (NPC) results from impaired intracellular transport of cholesterol and lipids, leading to neuronal loss. More recently, we began work on Juvenile Batten disease caused by mutation in \textit{CLN3}, a gene that encodes a transmembrane protein of unknown function. Our research group uses basic, translational, and clinical research approaches with the ultimate goal of developing and testing therapeutic interventions for rare genetic disorders. Our basic research uses neuronal, zebrafish, and mouse models of these genetic disorders to understand the biochemical, molecular, cellular, and developmental processes that underlie the birth defects and clinical problems encountered in affected patients. Our clinical research focuses on translating basic findings to the clinic. Natural history trials of SLOS, CLN3, and NPC1 are ongoing. We have large collections of biomaterial from well-characterized patients that can be used for biomarker discovery. Our emphasis on both basic and clinical research allows us to integrate laboratory and clinical data in order to improve our understanding of the pathological mechanisms underlying SLOS, CLN3, and NPC, with the goal of improving clinical care of these patients. Therapeutic trials have been conducted for SLOS and NPC1. Also, in collaboration with NCATS (the National Center for Advancing Translational Sciences), our research group has been involved in a multicenter trial of creatine transporter deficiency.

Inborn errors of cholesterol synthesis

SMITH-LEMLI-OPITZ SYNDROME (SLOS)

SLOS is an autosomal recessive, multiple-malformation syndrome characterized by dysmorphic facial features, cognitive impairment, hypotonia, poor growth, and various structural anomalies of heart,
lungs, brain, limbs, gastrointestinal tract, and genitalia. The SLOS phenotype is extremely variable. At the severe end of the phenotypic spectrum, infants often die as result of multiple major malformations, while mild SLOS combines minor physical malformations with behavioral and learning problems. The syndrome is attributable to an inborn error of cholesterol biosynthesis that blocks the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol.

Our laboratory initially cloned the human 3beta-hydroxysterol delta 7-reductase gene (DHCR7) and demonstrated mutations of the gene in SLOS patients. Together with others, we have so far identified over 100 mutations of DHCR7. We also used gene targeting in murine embryonic stem cells to produce several SLOS mouse models, including a null deletion and a hypomorphic point mutation. Mouse pups homozygous for the null mutation (Dhcr7delta3–5/delta3–5) exhibit variable craniofacial anomalies, are growth-retarded, appear weak, and die during the first day of life because they fail to feed. Thus, we were not able to use them to study postnatal brain development, myelination, or behavior or to test therapeutic interventions. For this reason, we developed a missense allele (Dhcr7T93M). The T93M mutation is the second most common mutation found in SLOS patients. Dhcr7T93M/T93M and Dhcr7T93M/delta3–5 mice are viable and demonstrate SLOS with a gradient of biochemical severity (Dhcr7delta3–5/delta3–5 greater than Dhcr7T93M/delta3–5 greater than Dhcr7T93M/T93M). We used Dhcr7T93M/delta3–5 mice to test the efficacy of therapeutic interventions on tissue sterol profiles. As expected, dietary cholesterol therapy improved the sterol composition in peripheral tissues but not in the central nervous system. Treatment of mice with the statin simvastatin improved the biochemical defect in both peripheral and central nervous system tissue, suggesting that simvastatin therapy may be used to treat some of the behavioral and learning problems in children with SLOS. Most recently, we developed a zebrafish model for SLOS that will allow us to study the impact of aberrant cholesterol synthesis on behavior.
Characterization of induced pluripotent stem cells from SLOS patients demonstrated a defect in neurogenesis, which results from inhibition of Wnt signaling owing to a toxic effect of 7-DHC.

As part of our clinical studies on SLOS, we identified a novel oxysterol, 27-hydroxy-7-dehydrocholesterol (27-7DHC), derived from 7-DHC in SLOS patients. We therefore investigated whether 27-7DHC contributes to the pathology of SLOS and found a strong negative correlation between plasma 27-7DHC and cholesterol levels in these patients. In addition, previous work showed that low cholesterol levels impair hedgehog signaling (a signaling pathway required for proper cell differentiation). Therefore, we hypothesized that increased 27-7DHC levels would have detrimental effects during development as a result of suppression of cholesterol levels. To test our hypothesis, we produced SLOS mice (Dhcr7$^{\delta 3-5/\delta 3-5}$) expressing a CYP27 (sterol 27-hydroxylase) transgene. These CYP27Tg mice display increased CYP27 expression and elevated 27-hydroxycholesterol levels but normal cholesterol levels. While Dhcr7$^{\delta 3-5/\delta 3-5}$ mice are growth-retarded, exhibit a low incidence of cleft palate (9%), and die during the first day of life, Dhcr7$^{\delta 3-5/\delta 3-5}$:CYP27Tg embryos are stillborn and have multiple malformations, including growth retardation, micrognathia, cleft palate (77%), lingual and dental hypoplasia, ankyloglossia, umbilical hernia, cardiac defects, cloacae, curled tails, and limb defects; we also observed autopod defects (polydactyly, syndactyly, and oligodactyly) in 77% of the mice. Consistent with our hypothesis, sterol levels were halved in the liver and 20-fold lower in the brain tissue of Dhcr7$^{\delta 3-5/\delta 3-5}$:CYP27Tg than in Dhcr7$^{\delta 3-5/\delta 3-5}$ embryos. The fact that 27-7DHC plays a role in SLOS may explain some of the phenotypic variability and may lead to development of a therapeutic intervention. The project is a good example of the benefits of integrating clinical and basic science to both understand the pathology of SLOS and develop potential therapeutic interventions. We are currently investigating the pathological role of other 7-DHC-derived oxysterols, such as DHCEO (3beta,5alpha-dihydroxy-cholest-7-en-6-one).
Development of patient-derived induced pluripotent stem cells has given us insight into fundamental mechanisms that impair neuronal development in SLOS.

We are conducting a longitudinal Natural History trial. Given that SLOS patients have a cholesterol deficiency, they may be treated with dietary cholesterol supplementation. To date, we have evaluated over 120 SLOS patients.

One reason for studying rare genetic disorders is to gain insight into more common disorders. Most patients with SLOS exhibit autistic characteristics. We are currently collaborating with other NIH and extramural groups to further evaluate this finding.

**LATHOSTEROLOSIS AND DESMOSTEROLOSIS**

Lathosterol 5-desaturase catalyzes the conversion of lathosterol to 7-dehydrocholesterol, representing the enzymatic step immediately preceding the defect in SLOS. Thus, to gain a deeper understanding of the roles of reduced cholesterol versus elevated 7-dehydrocholesterol levels in SLOS, we disrupted the mouse lathosterol 5-desaturase gene (Sc5d) by using targeted homologous recombination in embryonic stem cells. Sc5d<sup>−/−</sup> pups are stillborn, present with micrognathia and cleft palate, and exhibit limb-patterning defects. Many of the malformations in the mutant mice resemble malformations in SLOS and are consistent with impaired hedgehog signaling during development. Biochemically, the mice exhibit markedly elevated lathosterol levels and reduced cholesterol levels in serum and tissue.

Desmosterolosis is another inborn error of cholesterol synthesis that resembles SLOS. It results from a mutation in **DHCR24**, the gene encoding 3beta-hydroxysterol delta 24-reductase. DHCR24 catalyzes the reduction of desmosterol to cholesterol. We disrupted the mouse Dhcr24 gene with targeted homologous...
recombination in embryonic stem cells. Surprisingly, although most Dhcr24 mutant mice die at birth, the pups are phenotypically normal.

Niemann–Pick disease type C1

Niemann-Pick disease type C1 (NPC1) is a neurodegenerative disorder that results in ataxia and dementia. In view of the dementia, it has been referred to as childhood Alzheimer disease. The disorder is caused by a defect in intracellular lipid and cholesterol transport. Initially, as part of a bench-to-bedside award, we began a clinical protocol to identify and characterize biomarkers that could be used in a subsequent therapeutic trial. The project also received support from the Ara Parseghian Medical Research Foundation and Dana’s Angels Research Trust. We enrolled over 130 NPC1 patients in a longitudinal Natural History trial. The goals of the trial are to identify: (1) a blood-based diagnostic/screening test; (2) biomarkers that can be used as tools to facilitate development and implementation of therapeutic trials; and (3) clinical symptoms/signs that may be used as efficacy outcome measures in a therapeutic trial.

Currently, the average time from first symptom to diagnosis, the ‘diagnostic delay,’ in our cohort of NPC patients is on the order of four to five years. In collaboration with Daniel Ory, we found elevated levels of non-enzymatically produced oxysterols in NPC1 patients. Testing for oxysterols or bile acid derivatives has now become a standard method of diagnosis, and they are a potential biomarker that may be used to follow therapeutic interventions. We are now involved in a collaboration to implement newborn screening for NPC.

In addition to our Natural History study, we completed a randomized, placebo-controlled, cross-over trial to investigate the safety and efficacy of N-acetyl cysteine (NAC) in NPC1. The goal was to determine whether NAC treatment would reduce oxidative stress and subsequently lower levels of the non-enzymatically produced oxysterols. We also tested the safety and efficacy of the histone deacetylase (HDAC) inhibitor
vorinostat in adult NPC1 patients. In collaboration with the Therapeutics of Rare and Neglected Disease Program of NCATS, we completed a phase 1/2a therapeutic trial of lumbar intrathecal cyclodextrin (VTS-270, admabetadex) therapy in NPC1. We have now transitioned to a multicenter, multinational phase 2b/3, which is being evaluated. We have undertaken a study to evaluate the safety and efficacy of combined intrathecal and intravenous cyclodextrin and are collaborating with investigators at St. Louis Children’s Hospital to study the efficacy of cyclodextrin to ameliorate liver disease in infants with NPC.

To complement the clinical work, we have begun to apply molecular and proteomic approaches to both mouse and human biomaterials in order to identify biological pathways disrupted in NPC1. We identified several blood and CSF (cerebral spinal fluid) proteins and are in the process of validating the biomarkers as potential outcome measures to be used as tools in the development of therapeutic interventions.

**Creatine transport deficiency and CLN3 Disease**
Recently, we initiated natural history protocols to study children with creatine transport deficiency (CTD) and CLN3 disease (juvenile Batten disease). CTD is an X-linked disorder arising from mutation of \( SLC6A8 \) (which encodes Solute Carrier Family 6 Member 8, a protein called sodium- and chloride-dependent creatine transporter 1). Individuals with CTD manifest significant developmental delay and have frequent seizures. The work on CTD is a multicenter trial being conducted in collaboration with NCATS and Lumos Pharma. Our goal is to obtain detailed natural history data, establish a biorepository, find biomarkers, and identify potential clinical outcome measures in preparation for a therapeutic trial.

CLN3 disease (juvenile Batten disease) is an autosomal recessive, progressive neurodegeneration arising from mutation of \( CLN3 \), the gene encoding the lysosomal/endosomal protein battenin. The function of the battenin is not known, but its absence leads to a lysosomal storage disorder. Children with CLN3 disease typically first lose vision, followed by progressive cognitive and motor impairment. Similar to the other disorders that we study, our goal is to conduct a natural history study in order to facilitate studies designed to understand the pathology underlying these disorders as well as to develop therapeutic interventions.

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- Mallinckrodt CRADA
- Amicus CRADA
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- NICHD Strategic Plan Award
Publications


Collaborators

- William Balch, PhD, The Scripps Research Institute, La Jolla, CA
- Elizabeth Berry-Kravis, MD, PhD, Rush University Medical Center, Chicago, IL
- Juan Bonifacino, PhD, Section on Intracellular Protein Trafficking, NICHD, Bethesda, MD
- Stephanie Cologna, PhD, University of Illinois at Chicago, Chicago, IL
- Patricia Dickson, MD, St. Louis Children's Hospital, Washington University School of Medicine, St. Louis, MO
- Judith Miller, PhD, Children's Hospital of Philadelphia, Philadelphia, PA
- Daniel Ory, MD, Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO
- William J. Pavan, PhD, Genetic Disease Research Branch, NHGRI, Bethesda, MD
- Claire Le Pichon, PhD, Unit on the Development of Neurodegeneration, NICHD, Bethesda, MD
- Ninet Sinaii, PhD, Biostatistics and Clinical Epidemiology Service, NIH Clinical Center, Bethesda, MD
- David Sleat, PhD, Center for Advanced Biotechnology and Medicine, Rutgers University, Piscataway, New Jersey
- Beth Solomon, MS, CCC-SLP, Rehabilitation Medicine Department, NIH Clinical Center, Bethesda, MD
- Audrey Thurm, PhD, Neurodevelopmental and Behavioral Phenotyping Service, NIMH, Bethesda, MD
- Charles H. Vite, DVM, PhD, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA
- Michael E. Ward, MD, PhD, Inherited Neurodegenerative Diseases Unit, NINDS, Bethesda, MD
- Wei Zheng, PhD, Therapeutics for Rare and Neglected Diseases Program, NCATS, Bethesda, MD

Contact

For more information, email fdporter@helix.nih.gov or visit https://irp.nih.gov/pi/forbes-porter.
Three-Dimensional Organization of the Genome as a Determinant of Cell-Fate Decisions

Our lab seeks to understand cell-lineage differentiation, gene regulation, and how non-coding DNA elements and the 3D architecture of chromosomes contribute to these processes during development and disease. We are also interested in early mammalian development as a system in which to decipher how cells make lineage decisions and how gene-regulatory networks are established.

Eukaryotic cells need to deal with the biophysical constraints of packaging two meters of DNA inside a tiny nucleus (2–10 microns) and still retain the ability to access both its coding and non-coding elements to precisely orchestrate gene expression programs. Research over the past decade has begun to elucidate the mechanisms through which DNA condensation and organization in the nucleus are achieved. The results of such research suggest that the processes are tightly controlled and are themselves critical components of gene regulation. Our long-term goal is to understand how such processes occur in vivo and how their regulation dictates cell identity and cell-fate decisions in mammals.

To do so, we combine the robustness of mouse-genome editing and genetics with cutting-edge sequencing-based genomic techniques such as ATAC-seq (assay for transposase-accessible chromatin using sequencing), ChIP-seq (chromatin-immuno-precipitation DNA-sequencing), and Hi-C (high-throughput chromosome conformation capture technique), as well as live-imaging approaches. We believe that the early mouse embryo is an ideal model system in which to determine how nuclear architecture is regulated in the context of an organism and how that impacts cell behavior and identity.

Fertilization is the ultimate reprogramming experiment, where two highly differentiated cells (oocyte and sperm) fuse to form a zygote with totipotent potential. This involves a massive rearrangement of epigenetic modifications, both at the level of the DNA and of the histones, and the activity of many transcriptional regulators. Our studies aim to understand how 3D chromatin structures are established during this period and how they impact future developmental decisions.

Following fertilization and within a few cell divisions, the first cell lineages are established and different gene-expression programs are
FIGURE 1. Representative image of the lab’s research

We combine imaging techniques in both fixed and living cells with sequencing-based genomic techniques that assess DNA–DNA interactions.

A. Hi-C and CTCF ChIP-seq of GM1278 cells, which allow characterization of chromatin structure and identification of binding sites of an important architectural protein.

B. dCAS9 MCP-EGFP and PCP-CHERRY live imaging of the Igh and Akap6 loci. The mouse embryo is an unparalleled system in mammalian biology for understanding how tissue-specific gene expression is achieved.

C. Whole mount in situ hybridization for patterning markers in mid and late gastrulating embryos

D. Tetraploid aggregation with GFP (green fluorescent protein)–labeled ES (embryonic stem) cells allows generation of fully ES–cell derived embryos.

Put into action. In mammals, the result is the formation of the blastocyst, a structure that contains three different cell types, each with a defined differentiation potential. The trophectoderm is responsible for forming the placenta, the primitive endoderm leads to the yolk sac, and the epiblast gives rise to all remaining embryonic tissues. We will build on decades of lineage-fate experiments and precisely characterized signaling pathways known to regulate early mouse development to understand the contribution of nuclear organization to gene regulation during these early cell fate decisions.

We are also interested in understanding not only how DNA organization impacts cell behavior, and ultimately animal development and health, but also the mechanisms through which DNA folding itself is established and regulated, and which proteins are involved in these processes. To broadly address such questions, we will employ several high-throughput technologies that we have established in the lab, in combination with genome-wide CRISPR screens. Ultimately, we will fully characterize in vivo candidates identified this way to stringently determine their impact on gene regulation during mammalian development.

Publications


**Collaborators**

- Sevinc Ercan, PhD, *New York University, New York, NY*
- Stefan Feske, MD, *New York University School of Medicine, New York, NY*
- Daniel Herranz, PharmD, PhD, *Cancer Institute of New Jersey, Rutgers University, New Brunswick, NJ*
- Timothy Petros, PhD, *Unit on Cellular and Molecular Neurodevelopment, NICHD*
- Danny Reinberg, PhD, *New York University School of Medicine, New York, NY*
- Achim Werner, PhD, *Stem Cell Biochemistry Unit, NIDCR, Bethesda, MD*

**Contact**

For more information, email *pedro.rocha@nih.gov* or visit [http://rochalab.nichd.nih.gov](http://rochalab.nichd.nih.gov).
Mechanisms of Disease in Preterm Labor and Complications of Prematurity; Prenatal Diagnosis of Congenital Anomalies

Preterm birth is the leading cause of perinatal morbidity and mortality worldwide. The cost of prematurity in the U.S. alone is estimated to be $26 billion per year. An important goal is to understand the mechanisms of disease responsible for spontaneous preterm birth and fetal injury and to develop methods for the prediction and prevention of preterm birth.

The Perinatology Research Branch (PRB) proposed that preterm parturition is a syndrome caused by many pathologic processes, i.e., that preterm labor is one syndrome but has many causes. The emphasis of our Branch is to study intra-amniotic infection and inflammation, vascular disorders, maternal anti-fetal rejection (chronic inflammatory lesions of the placenta), cervical disease, and a decline in progesterone action. Previously, we reported that intra-amniotic inflammation, which affects at least one out of every three preterm neonates, is characterized by the activation of amniotic-fluid neutrophils, cells that represent the first line of defense against infection. Using DNA fingerprinting, we determined that amniotic-fluid neutrophils are of fetal origin in cases of preterm labor, maternal origin in cases of clinical chorioamnionitis at term, and mixed origin in patients who have inflammatory processes near term. Moreover, in a series of studies, we were able to demonstrate that neutrophils produce anti-microbial peptides and exhibit the formation of extracellular traps, whereby they immobilize and kill bacteria.

The Branch also studies other obstetrical syndromes that account for the high rate of infant mortality in the United States, including clinical chorioamnionitis, which is the most common infection-related diagnosis in delivery units around the world, as well as meconium aspiration syndrome and amniotic fluid embolism.

Congenital anomalies continue to be a leading cause of perinatal mortality in the U.S. Imaging, a powerful tool for scientific discovery, has changed the practice of obstetrics and maternal-fetal medicine. Imaging with ultrasound permits the definition of fetal anatomy, biometry, growth, and the study of physiologic parameters, such as cardiac function, fetal sleep, and breathing. We reported that fetal intelligent navigation echocardiography (FINE) can be used to screen for congenital heart disease and we addressed the frequency
Regulatory T cells and adverse neonatal outcomes

Regulatory T cells emerge in placental mammals to enforce maternal-fetal tolerance; therefore, most research has focused on understanding their role during early pregnancy. However, their role in the third period of gestation had not been mechanistically investigated. We undertook an extensive investigation, which included both human and animal models, to identify a role for regulatory T cells in the pathophysiology of preterm labor/birth and adverse neonatal outcomes. First, we showed that women with idiopathic preterm labor/birth had reduced proportions of functional regulatory T cells at the maternal-fetal interface. Next, we showed that the depletion of regulatory T cells led to preterm birth in a subset of cases. More importantly, the depletion led to growth restriction in both preterm and term neonates, deleterious effects that were ameliorated by the restoration of such cells to the mother. The immune mechanisms implicated in adverse perinatal outcomes induced by the depletion of regulatory T cells involved: first, a mild systemic inflammatory response mediated by CCL7 and IL-22; second, specific innate and adaptive immune responses in the decidua, myometrium, and placenta; and third, dysregulation of developmental and cellular processes in the placenta; these all occurred in the absence of intra-amniotic inflammation. The study represents the first mechanistic evidence supporting a role for regulatory T cells in spontaneous preterm labor and birth as well as impaired growth of the offspring (Figure 1).
Prevention of preterm birth and neonatal mortality with clarithromycin

One of every four preterm neonates is born to women with intra-amniotic infection, a clinical condition commonly associated with invasion of the amniotic cavity by *Ureaplasma* species. Yet, little is known about the taxonomy of and host immune response against these bacteria. We applied a multifaceted approach, including human amniotic fluid samples, *in vivo* models, and *in vitro* manipulations, to study the maternal-fetal immunobiology of *Ureaplasma* infection during pregnancy and a strategy to treat this condition. First, we performed a taxonomic characterization of *Ureaplasma* isolates from women with intra-amniotic infection, which revealed that *Ureaplasma parvum* is the most common bacterium found in this clinical condition. Next, we showed, in using animal models, that the intra-amniotic inoculation of *Ureaplasma* isolates induced varying rates of preterm birth and, more importantly, induced high rates of mortality in preterm and term neonates. Regardless of their potency to induce preterm birth, *Ureaplasma* isolates were capable of inducing a severe inflammatory response in the amniotic cavity and fetus. Notably, treatment with clarithromycin, a recently recommended yet not widely utilized antibiotic, prevented preterm birth and neonatal mortality induced by the intra-amniotic inoculation of *Ureaplasma parvum*. The investigations give insight into the maternal-fetal immunobiology of intra-amniotic infection and provide a critical demonstration of the effectiveness of clarithromycin to treat this clinical condition (Figure 2).

The efficacy of cervical pessary to prevent preterm birth

We performed a meta-analysis to assess the efficacy and safety of cervical pessary to prevent preterm birth and adverse perinatal outcomes in asymptomatic high-risk women. A total of 12 studies involving 4,687 women and 7,167 fetuses/infants were included in the review. Overall, there were no significant differences between the pessary and no-pessary groups in the risk of spontaneous preterm birth at less than 34 weeks of gestation among singleton gestations with a short cervix (cervical length of 25 mm or less), unselected twin gestations, twin gestations with a cervical length of less than 38 mm, and twin gestations with a cervical length of 25 mm or less. Overall, no significant differences were observed between the pessary and no-pessary groups in preterm birth at less than 37, 32, or 28 weeks of gestation, and most adverse pregnancy, maternal, and perinatal outcomes. Vaginal discharge was significantly more frequent in the pessary group than in the no-pessary group. In conclusion, current evidence does not support the use of cervical pessary to prevent preterm birth or to improve perinatal outcomes in singleton or twin gestations with a short cervix and in unselected twin gestations.
Single-cell transcriptional signatures of the human placenta

Understanding human parturition is essential to tackle the challenge of prematurity, which affects 15 million neonates every year. We investigated the human placenta at single-cell resolution in spontaneous labor at term and in preterm labor, having completed the first molecular characterization of the common pathway of preterm and term parturition. The key observations are as follows. First, we characterized, for the first time, the common molecular pathway of human parturition (spontaneous term labor and preterm labor) at single-cell resolution, where most of the differentially expressed genes were found in maternal macrophages from the chorioamniotic membranes and were involved in immune and inflammation-related pathways (Figure 3). Second, in the context of preterm labor, we found that most differentially expressed genes were detected in trophoblast cell types. Third, we identified two new cell types: maternal lymphatic endothelial decidual cells in the chorioamniotic membranes; and non-proliferative interstitial cytotrophoblasts in the placental villi (Figures 3A and 3B). Fourth, we identified placental scRNA-Seq transcriptional signatures in the maternal circulation, revealing that the cellular dynamics of the placenta can be reflected in maternal blood total RNA and, therefore, can be monitored throughout pregnancy (Figure 3C).

The human placenta and SARS-CoV-2

Using single-cell gene expression, we determined that co-expression of the ACE2 and TMPRSS2 transcripts is negligible in the placenta and chorioamniotic membranes. ACE2 and TMPRSS2 are the major proteins used by SARS-CoV-2 to infect the cells, spread, and cause COVID-19 disease; yet, they are rarely co-transcribed in
any of the cell-types of the placenta or at any of the time points spanning the three trimesters of pregnancy. In contrast, the transcripts for receptors of other viruses known to cause fetal infection, such as Zika and cytomegalovirus, were found in larger quantities in placental cells. The finding helps to explain why SARS-CoV-2 is rarely vertically transmitted from mother to fetus.

The cellular transcriptome in the maternal circulation during pregnancy

Pregnancy represents a unique immunological state, in which the mother adapts to tolerate the semi-allogenic conceptus; yet, the cellular dynamics in the maternal circulation are poorly understood. Using exon-level expression profiling of up to six longitudinal whole blood samples from 49 pregnant women we found that: (1) the strongest expression changes followed three distinct longitudinal patterns, with genes related to host immune response (e.g., MMP8, DEFA1B, DEFA4, LTF) showing a steady increase in expression from 10 to 40 weeks of gestation (see red cluster in Figure 4); (2) many biological processes and pathways related to immunity and inflammation were modulated during gestation; (3) the expression of mRNA signatures of T cells, B cells, and erythroid cells, defined using single-cell genomics, followed unique patterns during gestation; and (4) significant whole-blood mRNA and plasma protein correlations were observed for genes that are part of the T cell signature. The findings provide insight into the immunobiology of normal pregnancy and suggest candidate predictors for dating gestational age.

New and advanced features of fetal intelligent navigation echocardiography (FINE)

Congenital heart disease (CHD) is the leading organ-specific birth defect, as well as the leading cause of infant
morbidity and mortality from congenital malformations. Therefore, a comprehensive screening examination of the fetal heart should be performed in all women to maximize the detection of CHD. Four-dimensional sonography with spatiotemporal image correlation (STIC) technology displays a cine loop of a complete single cardiac cycle in motion. A novel method known as fetal intelligent navigation echocardiography (FINE) was previously developed to interrogate STIC volume datasets using intelligent navigation technology. The method permits the automatic display of nine standard fetal echocardiography views, required to diagnose most cardiac defects. FINE considerably simplifies fetal cardiac examinations and reduces operator dependency. It has both high sensitivity and specificity for the detection of CHD. Indeed, FINE has been integrated into several commercially available ultrasound platforms. Recently, eight novel and advanced features were developed for the FINE method. Such features can be categorized based upon their broad goals. The first goal is to simplify FINE further, and consists of the following features: (1) auto fetal positioning (or FINE align); (2) skip points (to allow quantitative measurements to be performed on the cardiac views generated by FINE); (3) predictive cursor; (4) static mode volume (Figure 5); (5) breech sweep; (6) automatic cardiac axis; and (7) cardiac biometry. The last goal is to improve the success of obtaining fetal echocardiography view(s) and consists of (8) Maestro planar navigation.

Optical ultrasound simulation–based training in obstetric sonography
Ultrasound is an imaging modality that is highly operator dependent. This year, we published a review of the challenges in learning how to perform obstetric sonography and the processes necessary to acquire expert performance skills in sonography; we evaluated simulation-based education and learning, and the value of medical simulation. Ultrasound simulators are an effective means of teaching obstetric sonography, because it provides training, deliberate practice, and performance evaluation/feedback, which allows continuous and critical self-evaluation. We reviewed evidence that simulation can improve performance in obstetric ultrasound examination, reviewed current simulators, and discussed the current problems/gaps in ultrasound simulation. Optical positioning ultrasound simulation is a novel high-fidelity simulation learning system, which addresses
A four-dimensional spatiotemporal image correlation volume dataset has been loaded onto the computer. The computer screen depicts the following: (1) target plane (small image of four-chamber view) located on the left side of the screen; (2) plane being generated by the virtual transducer (large image of the five-chamber view); (3) visual graphic (left upper corner) provides instructions via contextual video and animation on how to move the virtual transducer to achieve the target plane; (4) inclinometer (upper right panel showing three diagrams); and (5) cube (lower right panel).

many of these problems/gaps, and we introduced this for the first time in this article. The technology uses a camera-based optical tracking system and has a limited amount of hardware, making the simulator compact, portable, and personal. A computer or laptop is used to load actual (vs. artificially created) ultrasound cases, which are organ-specific. The goal of the trainee is to achieve a sonographic target plane. The Guided mode (Figure 6) uniquely provides simulator voice commands that instruct the user on how to manipulate the transducer, contextual videos and animation, and graphic feedback to guide the user towards obtaining the correct target plane of interest (e.g., four-chamber view).

The amniotic fluid cell-free transcriptome

The amniotic fluid (AF) cell-free RNA (cfRNA) is modulated with physiologic and pathologic processes during pregnancy. We therefore sought to define AF normal expression and splicing patterns to aid in identifying biomarkers for assessing fetal development and obstetrical disease. About one hundred AF transcriptomes were profiled in samples collected during midtrimester and at term, and we quantified the expression of tissue-specific and cell-type-specific signatures defined by single-cell genomics. We found that 11% of the coding and 6% of the non-coding AF genes were differentially expressed between midtrimester and term gestation. Expression changes with advancing gestation featured increased expression of genes specific to the trachea, salivary glands, and lung; and decreased expression of genes specific to the cardiac myocytes, uterus, and fetal liver, among others. We also found that, with advancing gestation, differential splicing involved genes related to brain development and immunity pathways, including some that were missed based on differential expression analysis alone. This is thus the largest AF transcriptomics study in normal pregnancy, reporting for the first time that single-cell genomic signatures can be tracked in the AF and display complex patterns of expression during gestation. We also demonstrated a role for alternative splicing in tissue-identity acquisition, organ development, and immune processes. The results may have implications for the development of fetal testing to assess placental function and fetal organ maturity (Figure 7).
FIGURE 7. Amniotic fluid cell-free transcriptome in normal pregnancy

Principal components analysis (left); tissues and cell-type signatures with increasing (center); and decreasing (right) expression patterns with advancing gestational age.

Publications


Collaborators

- Tinnakorn Chaiworapongs, MD, Wayne State University School of Medicine, Detroit, MI
- Agustin Conde-Agudelo, MD, Wayne State University School of Medicine, Detroit, MI
- Mark Haacke, PhD, Wayne State University School of Medicine, Detroit, MI
- Leonid Margolis, PhD, Section on Intercellular Interactions, NICHD, Bethesda, MD
- Adi L. Tarca, PhD, Wayne State University, Detroit Medical Center, Detroit, MI
• Lami Yeo, MD, Wayne State University School of Medicine, Detroit, MI
• Bo Hyun Yoon, MD, PhD, Seoul National University, Seoul, Korea

Contact
For more information, email romeror@mail.nih.gov or visit http://irp.nih.gov/pi/roberto-romero.
Regulation of Mammalian Intracellular Iron Metabolism and Biogenesis of Iron-Sulfur Proteins

Our goal is to understand how mammals regulate intracellular and systemic iron metabolism to support processes that require iron and iron-sulfur clusters. Iron-regulatory proteins 1 and 2 (IRP1 and IRP2) regulate the expression of numerous proteins involved in iron metabolism. In iron-depleted cells, the proteins bind to RNA stem-loops in transcripts known as iron-responsive elements (IRE). IRP binding stabilizes the mRNA that encodes the transferrin receptor and represses the translation of transcripts that contain IREs near the 5' end of the ferritin H and L chains. IRP1 is an iron-sulfur protein that functions as an aconitase in iron-replete cells. IRP2 is homologous to IRP1 but undergoes iron-dependent degradation in iron-replete cells. In mouse models, loss of IRP2 results in mild anemia, erythropoietic protoporphyria, and adult-onset neurodegeneration, all the likely result of functional iron deficiency. Biochemically and using expression arrays, we studied, in Irp2−/− mice, the mechanisms that lead to anemia and neurodegeneration with motor neuron loss. We are using our mouse model of neurodegeneration to identify compounds that can prevent neurodegeneration; for example, we found that the antioxidant Tempol works by activating the latent IRE-binding activity of IRP1. Given that mitochondrial energy production is required to maintain axonal integrity and that motor neurons have the longest and most vulnerable axons, we hypothesize that mitochondrial dysfunction resulting from iron deficiency causes axonal degeneration. We discovered that deficiency in IRP1 causes polycythemia and pulmonary hypertension resulting from translational derepression of hypoxia-inducible factor (HIF) 2a through the IRE–IRP system. Our discovery introduces a new level of physiological regulation of erythropoiesis and provides a model for early pulmonary hypertension.

Our ongoing work on iron-sulfur cluster biogenesis has led to new insights into how mammalian iron-sulfur clusters are synthesized and transferred to appropriate recipient proteins. Several human diseases are now known to be caused by deficiencies in the iron-sulfur cluster biogenesis machinery. We developed a treatment for the rare disease ISCU (iron-sulfur cluster assembly enzyme) myopathy. By identifying a tripeptide motif common to many iron-sulfur recipient proteins, we developed an algorithm that facilitates discovery of previously unrecognized mammalian iron-sulfur proteins. Our work suggests
that there are hundreds of previously unrecognized mammalian iron-sulfur proteins. Discovery of iron-sulfur cofactors will lead to breakthroughs in several research areas involving DNA repair, ribosomal biogenesis, mRNA translation, intermediary metabolism, and the regulation of the growth and energy-sensing pathways that are critical for determining the fates of many cell types.

The molecular basis for the regulation of intracellular iron metabolism in mammals

In previous years, our laboratory identified and characterized the cis and trans elements mediating iron-dependent alterations in the abundance of ferritin and the transferrin receptor. IREs are RNA stem-loops found in the 5′ end of ferritin mRNA and the 3′ end of transferrin receptor mRNA. We cloned, expressed, and characterized IRP1 and IRP2, two essential iron-sensing proteins. IRPs bind to IREs when iron levels are depleted, resulting in either inhibition of translation of ferritin mRNA and of other transcripts that contain an IRE in the 5′ untranslated regions (UTR) or stabilization of the transferrin receptor mRNA and possibly other transcripts that contain IREs in the 3′ UTR. The IRE-binding activity of IRP1 depends on the presence of an iron-sulfur cluster (see “Mammalian iron-sulfur cluster biogenesis” below). IRP2 also binds to IREs in iron-depleted cells but, unlike IRP1, in iron-replete cells it is selectively ubiquitinated and then degraded by the proteasome.

To approach questions about the physiology of iron metabolism, we generated loss-of-function mutations of IRP1 and IRP2 in mice through homologous recombination in embryonic cell lines. In the absence of provocative stimuli, we initially observed no abnormalities in iron metabolism associated with loss of IRP1 function. Irp2−/− mice develop a progressive neurologic syndrome characterized by gait abnormalities and axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. Ferritin overexpression occurs in affected neurons and in protrusions of oligodendrocytes into the space created by axonal degeneration. Irp2−/− animals develop iron-insufficiency anemia and erythropoietic protoporphyria. In animals that lack IRP1, IRP2 compensates for loss of IRP1’s regulatory activity in most cell types, but we discovered several cell types and accompanying phenotypes in which Irp2 expression cannot be sufficiently increased to compensate. Animals that lack both IRP1 and IRP2 die as early embryos. The adult-onset neurodegeneration of adult Irp2−/− mice is exacerbated when one copy of Irp1 is also deleted. Irp2−/− mice offer a unique example of spontaneous adult-onset, slowly progressive neurodegeneration; analyses of gene expression and iron status at various stages of disease are ongoing. Dietary supplementation with the stable nitroxide Tempol prevents neurodegeneration; the treatment appears to work by recruiting the IRE-binding activity of IRP1. We found that motor neurons were the most adversely affected neurons in Irp2−/− mice and that neuronal degeneration accounted for the gait abnormalities. In collaboration with Grace Yoon, we discovered two Irp2−/− patients who suffered from severe neurodegenerative disease in infancy and were bed-ridden or died as adolescents.

We discovered a form of the iron exporter ferroportin lacking the IRE at its 5′ end that is important in permitting iron to cross the duodenal mucosa in iron-deficient animals and in preventing developing erythroid cells from retaining high amounts of iron in iron-deficient animals. Our findings explain why microcytic anemia is usually the first physiological manifestation of iron deficiency in humans. Unexpectedly, we discovered that ferroportin is an abundant protein on mature red cells, where, as our work showed, it is needed to export free iron released from heme by oxidation. Using erythroid ferroportin knockout animals, we showed that absence of ferroportin results in accumulation of intracellular iron, increased oxidative stress, and reduced viability of cells in circulation.
We noted that more than 8% of African Americans carry this allele, which has the potential to cause tissue iron overload in liver and kidney, perhaps accounting for some of the morbidities to which African Americans are unusually predisposed.

We recently discovered that loss of IRP1 causes polycythemia and pulmonary hypertension through derepression of hypoxia-inducible factor 2-alpha (HIF2a) translation in the renal interstitium through the IRE–IRP system. We confirmed that overexpression of HIF2a drives production of erythropoietin and polycythemia in a mouse model of Chuvash polycythemia (an autosomal recessive form of erythrocytosis, which is endemic in patients from Chuvashia, an autonomous republic within the Russian Federation), and we discovered that we could reverse disease by activating Irp1 to repress HIF2a translation using TEMPOL, which converts Irp1 from the aconitase to the IRE–binding form. Phlebotomy has not been a very helpful therapy to the thousands of patients with Chuvash polycythemia in Russia, and we propose that oral Tempol supplementation could constitute a good therapeutic intervention. We also are conducting experiments with HIF2alpha inhibitors, which reveal that the drugs reverse polycythemia and pulmonary hypertension in our Irp1–/- and Chuvash polycythemia models.

We also elucidated the pathophysiology of intravascular hemolysis and hyposplenism in animals that lack heme oxygenase 1 (HMOX1). Their tissue macrophages die because they cannot metabolize heme after phagocytosis of red cells. To mitigate or reverse disease, we performed bone marrow transplants from wild-type animals to supply animals with functional macrophages, transplants that were successful. We then discovered that the transplant was not necessary by demonstrating that exogenously expanded wild-type macrophages can repopulate the reticuloendothelial system of Hmox1–/- mice, restore normal erythrophagocytosis, and reverse renal iron overload and anemia. Five human HMOX1–/- patients have been identified, but we believe this represents an underdiagnosed and often misdiagnosed rare human disease.

Upon realizing that ferroportin is key to reducing free iron levels in red cells, we analyzed the Q248H mutation of ferroportin, which confers gain of function and reduces iron abundance in red cells. The Q248H mutation underwent positive selection in malarious regions of Africa, and we hypothesized that it conferred resistance to malaria by diminishing iron available to support growth of the malaria parasite in red cells. Upon infecting mice that lacked erythroid ferroportin with several malaria strains, we demonstrated that the mice experienced increased morbidity and mortality, likely because iron concentrations in red cells were high and supported parasite growth well.
Mammalian iron-sulfur cluster biogenesis

Our goal in studying mammalian iron-sulfur biogenesis is to understand how iron-sulfur prosthetic groups are assembled and delivered to target proteins in the various compartments of mammalian cells, including mitochondria, the cytosol, and the nucleus. We also seek to understand the role of iron-sulfur cluster assembly in the regulation of mitochondrial iron homeostasis and in the pathogenesis of diseases such as Friedreich’s ataxia and sideroblastic anemia, which are both characterized by incorrect regulation of mitochondrial iron homeostasis.

IRP1 is an iron-sulfur protein related to mitochondrial aconitase, a citric acid cycle enzyme; it functions as a cytosolic aconitase in iron-replete cells. Regulation of the RNA–binding activity of IRP1 involves a transition from a form of IRP1 in which a [4Fe-4S] cluster is bound to a form that loses both iron and aconitase activity. The [4Fe-4S]–containing protein does not bind to IREs. Controlled degradation of the iron-sulfur cluster and mutagenesis reveal that the physiologically relevant form of the RNA–binding protein in iron-depleted cells is an apoprotein. The status of the cluster appears to determine whether IRP1 binds to RNA.

We identified numerous mammalian enzymes of iron-sulfur cluster assembly that are homologous to those encoded by the \textit{NIFS}, \textit{ISCU}, and \textit{NIFU} genes, which are implicated in bacterial iron-sulfur cluster assembly, and we observed that mutations in several iron-sulfur cluster biogenesis proteins cause disease. Loss of frataxin, a protein that promotes the biosynthesis of heme and assembly and repair of iron-sulfur clusters by enhancing early steps of iron-sulfur cluster biogenesis, causes Friedreich’s ataxia, which is characterized by progressive compromise of balance and cardiac function. In a cohort of patients of Swedish descent, we found that loss of the iron-sulfur cluster assembly enzyme ISCU causes skeletal myopathy. To explain the tissue specificity of the ISCU myopathy, we studied myoblasts and other patient-derived tissue samples and cell lines. We discovered that many factors contribute to insufficiency of ISCU in skeletal muscle, including more pronounced abnormal splicing and unusual sensitivity of ISCU to degradation upon exposure to oxidative stress. Thus, oxidative stress may impair the ability of tissues to repair damaged iron-sulfur clusters by directly damaging a key component of the biogenesis machinery. We discovered that antisense therapy would likely work as a treatment for ISCU myopathy patients, as we were able to correct the causal splicing defect in patient myoblasts using stable antisense RNAs that were manufactured by high-quality techniques suitable for use in patients. In one patient, we found that a splicing abnormality of glutaredoxin 5 was associated with sideroblastic anemia. In the affected tissues, mitochondrial iron overload is a feature common to all three diseases.

We identified a tripeptide motif, LYR, in apoproteins that are recipients of nascent iron-sulfur clusters. The co-chaperone HSC20 binds to HSPA9, its partner HSP70–type chaperone, and the chaperone complex binds to ISCU bearing a nascent iron-sulfur cluster and to iron-sulfur cluster–recipient proteins. We identified several direct iron-sulfur–recipient proteins in a yeast two-hybrid assay, using HSC20 as bait. By studying one known iron-sulfur recipient, succinate dehydrogenase subunit B (SDHB), we discovered that several LYR motifs of the SDHB primary sequence engage the iron-sulfur transfer apparatus by binding to the C-terminus of HSC20, facilitating delivery of the three iron-sulfur clusters of SDHB. We further discovered that the assembly factor SDHAF1 also engages the iron-sulfur cluster transfer complex to facilitate transfer of iron-sulfur clusters to SDHB. The discovery of the LYR motif will aid in the identification of unknown iron-sulfur proteins, which are likely to be much more common in mammalian cells than had been previously appreciated. More recently, we discovered that, through recognition of LYR–like motifs in these recipient proteins, HSC20 is responsible for the delivery of iron-sulfur clusters to respiratory chain complexes I–II. Using informatics, we predicted that
amino levulinic acid dehydratase (ALAD), a heme-biosynthetic enzyme, is a previously unrecognized iron-sulfur protein, and we identified more unrecognized iron-sulfur proteins by using the LYR motif to analyze candidate proteins.

Using informatics, over-expression of candidate proteins, and iron detection with ICP–MS (inductively coupled mass spectrometry), we identified many more iron-sulfur proteins that are involved in a wide range of metabolic pathways, ranging from intermediary metabolism, DNA repair, and RNA synthesis, and possibly regulation of cellular growth. Iron-sulfur proteins will prove to be integral to the functioning and sensing of numerous pathways important in cellular functions.

We discovered that the mitochondrial protein ABCB7 (ATP–binding cassette sub-family B member 7) forms a complex with dimeric ferrochelatase, which binds ABCB10 to the other half of the ferrochelatase dimer. Our preliminary results suggest that ABCB7 may represent a mitochondrial heme exporter.

We discovered that the intermediary scaffold protein NFU1 acquires its iron-sulfur clusters from ISCU2 and ISCA1 to form a cubane iron-sulfur cluster that is delivered directly to lipoic acid synthase. We are working to shed light on the complex use of secondary iron-sulfur scaffold proteins to deliver iron-sulfur cluster to many recipient proteins in the cell.

**Additional Funding**
- Bench-to-Bedside Award: Analysis of whether the Ferroportin Q248H mutation prevalent in Africans and African Americans predisposes to unrecognized pathological tissue iron overload and disease

**Publications**
Collaborators

- J. Martin Bollinger, PhD, Penn State University, University Park, PA
- Victor Gordeuk, MD, University of Illinois College of Medicine, Chicago, IL
- Carsten Krebs, PhD, Penn State University, University Park, PA
- W. Marston Linehan, MD, Urologic Oncology Branch, Center for Cancer Research, NCI, Bethesda, MD
- John F. Tisdale, MD, Molecular and Clinical Hematology Branch, NIDDK, Bethesda, MD
- Grace Yoon, MD, The Hospital for Sick Children, Toronto, Canada

Contact

For more information, email trou@helix.nih.gov or visit https://irp.nih.gov/pi/tracey-rouault.
Mechanisms of Synapse Assembly and Homeostasis

In our research we strive to understand the mechanisms of synapse assembly, plasticity, and homeostasis. The chemical synapse is the fundamental communication unit connecting neurons in the nervous system to one another and to non-neuronal cells and whose purpose is to mediate rapid and efficient transmission of signals across the synaptic cleft. Synaptic transmission forms the basis of the biological computations that underlie and enable our complex behavior. Crucial to this function is the ability of a synapse to change its properties, so that it can optimize its activity and adapt to the status of the cells engaged in communication and/or to the larger network comprising them. Consequently, synapse development is a highly orchestrated process coordinated by intercellular communication between the pre- and postsynaptic compartments and by neuronal activity itself. Our long-term goal is to elucidate the molecular mechanisms that regulate formation of functional synapses during development and which fine-tune them during plasticity and homeostasis. We focus on four key processes in synaptogenesis: (1) trafficking of components to the proper site; (2) organizing those components to build synaptic structures; (3) maturation of the synapse to optimize its activity; and (4) homeostatic mechanisms that restore synapse activity after various perturbations in the system. We address the molecular mechanisms underlying these processes using a comprehensive set of approaches that include genetics, biochemistry, molecular biology, super-resolution imaging, and electrophysiology recordings in live animals and reconstituted systems.

Because of its many advantages, we study these events in a powerful genetics system, *Drosophila melanogaster*, and use the neuromuscular junction (NMJ) as a model for glutamatergic synapse development and function. The fact that individual NMJs can be reproducibly identified from animal to animal and are easily accessible for electrophysiological and optical analysis makes them uniquely suited for in vivo studies on synapse assembly, growth, and plasticity. In addition, the richness of genetic manipulations that can be performed in *Drosophila* permits independent control of individual synaptic components in distinct cellular compartments. Importantly, the fly NMJ relies entirely on kainate-type receptors, a family of ionotropic glutamate receptors that impact synaptic transmission and neuronal excitability in the mammalian central nervous system.
Neto, an auxiliary subunit for glutamate receptors, is essential for synapse development.

Many neurological disorders are linked to defects in synaptogenesis; however, the initial clustering functions critical for the synaptic recruitment and stabilization of neurotransmitter receptors are poorly understood. At vertebrate or insect NMJs, prior to motor neuron arrival at its target muscle, the neurotransmitter receptors form small, nascent clusters on the muscle, which are distributed in the vicinity of future synaptic sites. Motor neuron arrival triggers the formation of large synaptic receptor aggregates and promotes expression of more receptors to permit synapse maturation and growth. In flies as in vertebrates, the neurotransmitter receptor clusters interact with the local cytoskeleton and other synaptic structures to maintain local density, which involves solving three fundamental problems common to all chemical synapses: (1) trafficking the components to the proper site; (2) organizing them to build synaptic structures; and (3) maturation and homeostasis of the synapse to optimize its activity. Recent advances, particularly from vertebrate ionotropic glutamate receptor (iGluR) biology, reveal that the solution to such problems entirely depends on the activity of a rich array of auxiliary subunits that associate with the receptors. These highly diverse transmembrane proteins associate with iGluRs at all stages of the receptor life-cycle and mediate the delivery of receptors to the cell surface, their subcellular distribution, synaptic recruitment, their endocytosis and turnover, association with various postsynaptic density (PSD) scaffolds, and importantly, their channel properties. iGluRs assembled from different subunits have strikingly different biophysical properties; their association with different auxiliary subunits increases this diversity even further.

The Drosophila NMJ utilizes at least six kainate receptor (KAR) subunits, which form two distinct postsynaptic complexes (type-A and type-B) that co-exist within individual postsynaptic densities (PSDs) and enable NMJ functionality and plasticity, and a presynaptic complex containing the presynaptic KAR KaiRID, which modulates basal neurotransmission. The postsynaptic KARs are heterotetrameric complexes composed of three shared subunits, GluRIIC, GluRIID, and GluRIIE, as well as either GluRIIA (type-A receptors) or GluRIIB (type-B). The shared subunits are required for animal viability as they are essential for iGluR synaptic recruitment and therefore NMJ assembly and function. Our previous studies identified Drosophila Neto as an obligatory subunit of fly NMJ iGluR complexes. Neto belongs to a family of highly conserved auxiliary proteins that share an ancestral role in the formation and modulation of glutamatergic synapses. Vertebrate Neto1 and Neto2 and Caenorhabditis elegans Neto/SOL-2 were recently shown to modulate the properties of selective iGluRs, mostly KARs. Neto1/Neto2 double knockout mice have defects in long-term potentiation and in learning and memory, but the underlying mechanisms are extremely difficult to study owing to the low abundance of such channels and the small currents they elicit. In contrast, we found that Drosophila utilizes Neto and KARs at the NMJ, a synapse essential for viability. Using live imaging we showed that Neto clusters at nascent NMJs at the time when iGluRs begin to accumulate and cluster. Similar to mutants lacking essential, shared iGluR subunits, neto null mutants are completely paralyzed and die as embryos, with the iGluRs scattered as small aggregates, away from the neuronal arbor. Importantly, Neto does not cluster at synaptic locations in the...
absence of iGluRs, indicating that Neto and iGluRs depend on each other for trafficking and stabilization at synaptic sites. Our studies demonstrate that Neto engages the iGluRs on the muscle membrane and traffic together to synaptic sites where they form clusters. By controlling the clustering and trafficking of functional iGluR complexes, Neto directly controls synapse assembly, organization and maintenance of PSDs, and synapse functionality.

**Neto-mediated intracellular interactions sculpt the postsynaptic iGluR fields.**

The Neto proteins are multi-domain transmembrane proteins with two extracellular CUB (for complement C1r/C1s, UEGF, BMP-1) domains followed by an LDLa (low-density lipoprotein receptor domain class A) motif. CUB domains are BMP–binding, protein-interaction domains that could promote aggregation. *Drosophila neto* encodes two isoforms, Neto-α and Neto-β, with different cytoplasmic domains, generated by alternative splicing. The cytoplasmic domains, both rich in putative phosphorylation motifs and docking sites, are highly divergent among Neto proteins across species, presumably reflecting cell/tissue specific roles. To characterize the functional domains of Neto, we generated truncated Neto variants and tested their cellular distribution and ability to rescue Neto function during development. In previous studies we had found that the extracellular part of Neto is required for apical targeting as well as for clustering of Neto/iGluR complexes at the NMJ. Thus, muscle expression of a Neto variant with no intracellular domain (Neto-ΔCTD) can rescue the iGluRs recruitment in *neto*<sup>null</sup> mutants. We found that Neto activities are restricted by an inhibitory prodomain, which must be removed by Furin-mediated proteolysis. When the prodomain cleavage is blocked, Neto is properly targeted to the muscle membrane and engages the iGluR complexes *in vivo* but fails to enable the incorporation of iGluRs in stable synaptic clusters. The recruitment of PSD components is partly attributable to Neto–mediated intracellular interactions.

Neto-β is the predominant Neto isoform at the larval NMJ [Reference 1]. Our developmental studies indicate that Neto-β controls the synaptic recruitment of iGluRs and of other postsynaptic components, such as P21–activating kinase (PAK), an important PSD component previously implicated in the stabilization of type-A receptors at postsynaptic sites. Type-A and type-B glutamate receptor complexes differ in their composition, function, and mechanisms of trafficking to the synapses. Using a *neto-β* allelic series, we found that Neto-β...
uses its cytoplasmic domain as an organizing platform to recruit iGluRs and PSD proteins that stabilize the synaptic type-A receptors, thus sculpting the postsynaptic composition.

Neto-α represents less than 10% of the total Neto pool at the larval NMJ. We found that loss of Neto-α has no detectable effect on postsynaptic iGluR levels or the relative type-A:type-B receptor ratio but it does influence the iGluR receptor field [Reference 1]. In flies, the PSD-95 ortholog Discs Large (Dlg) flanks the PSD/iGluR fields, marked by GluRIIC, an obligatory subunit of the postsynaptic iGluR complexes (Figure 1). The PSD/Dlg boundaries are well defined in control boutons but are no longer recognizable at neto-αnull NMJs. Furthermore, the 3D reconstructions of the boutons showed no overlap between GluRIIC and Dlg signals in controls but significant overlap in neto-αnull mutants. 3D-structured illumination microscopy (3D-SIM) and serial electron microscopy analyses also captured significantly enlarged (by 30%) receptor fields at neto-αnull NMJs in (Figure 2). Muscle overexpression of a neto-α transgene fully rescued the PSD size of neto-αnull synapses. Thus, postsynaptic Neto-α limits the postsynaptic receptor fields.

Presynaptic Neto-α and KaiRiD control basal neurotransmission.

Loss-of-function and genetic epistasis experiments uncovered an unexpected role for Neto-α in the control of basal neurotransmission. Intracellular recordings of spontaneous miniature excitatory junctional potentials (mEJPs) and evoked excitatory junctional potentials (EJPs) from neto-αnull muscle indicate normal miniature amplitudes, a result consistent with normal iGluR levels at neto-αnull NMJs. However, neto-αnull animals showed EJP amplitudes reduced by 46% [Reference 1]. Knockdown of Neto-α in the motor neurons but not in the muscle induced similarly reduced EJP amplitudes; conversely, neuronal but not muscle expression of neto-αnull transgenes rescued the EJP amplitudes at neto-αnull NMJs. The data indicate that presynaptic Neto-α is required in the motor neurons to ensure normal levels of basal neurotransmission.

Given that the presynaptic KaiRiD-containing complex was recently implicated in the control of basal neurotransmission, we examined whether Neto-α modulates KaiRiD synaptic distribution and function. We found that neto-αnull and KaiRiDnull single and double mutants have similar physiologic deficits. Also, neuronal expression of Neto-α and KaiRiD can rescue the corresponding mutants, but cannot compensate for each other, indicating that Neto-α and KaiRiD function together to control basal neurotransmission. We previously
demonstrated that a minimal Neto variant called Neto-deltaCTD, which contains the highly conserved domains shared by Neto-α and Neto-β (the extracellular CUB domains, LDLα motif, and the transmembrane part) but has no intracellular C-terminal domain, is both required and sufficient for the synaptic recruitment and function of postsynaptic iGluRs. Interestingly, neuronal Neto-deltaCTD rescued basal neurotransmission at neto-αnull NMJs. In unpublished studies, we found that Neto-α and Neto-deltaCTD increase the desensitization rates and open probability for the KaiRID channels, thus modulating the function of this auto-receptor.

Similar to the muscle, we found that Neto-α, not the iGluR subunits, appears to be the limiting component at presynaptic terminals. Neuronal overexpression of KaiRID has no effect on the baseline, whereas overexpression of Neto-α or Neto-deltaCTD proportionally increases the basal neurotransmission levels. Thus, the auto-receptor function and likely synaptic levels are tightly controlled by Neto-α.

**Neto-α is an effector of synapse homeostasis.**

In flies as in vertebrates, neuronal activity induces input-specific changes in the synaptic strength; at larval NMJ, the postsynaptic sensitivity is primarily modulated via synapse-specific recruitment of type-A (GluRIIA-containing) receptors. Robust homeostatic mechanisms keep synapses within an appropriate dynamic range, so that the evoked potentials measured in the muscle remain constant from embryo to third instar larvae; reduced postsynaptic sensitivities (i.e., reduced GluRIIA activity) trigger a compensatory increase in quantal content (QC), the number of vesicles released by the neuron, known as presynaptic homeostatic potentiation (PHP). To study this homeostatic response, we used two well-established paradigms: (1) chronic (developmental); and (2) acute (pharmacological) induction of PHP. Specifically, (1) removal of GluRIIA during development leads to reduced quantal size (mEJP) and triggers PHP (increased QC), a PHP response that is not detectable in neto-αnull;GluRIIA double mutants. Also, (2) application of sub-blocking concentrations of philanthotoxin (PhTx), a polyamine toxin derived from wasp venom, to semi-intact larval preparations triggers a fast reduction in quantal size and an increase in QC, so that basal neurotransmission recovers within minutes. PhTx reduces the quantal size at neto-αnull NMJs, but basal neurotransmission never recovers. Similar
to above, the PHP deficits in neto-α\textsuperscript{null} mutants phenocopy those of KaiRID\textsuperscript{null} mutants. However, neuronal overexpression of Neto-α rescues the PHP defects in neto-α\textsuperscript{null}/KaiRID\textsuperscript{null} double or KaiRID\textsuperscript{null} single mutants, indicating that Neto-α is both required and sufficient for the induction of the PHP response. Moreover, we found that Neto-deltaCTD cannot sustain any PHP response, indicating that the intracellular domain of Neto-α is critical for this function.

How does Neto-α enable such homeostatic compensatory response? We found no deficits in the presynaptic Ca\textsuperscript{2+} entry at neto-α\textsuperscript{null}–mutant NMJs. The absence of Neto-α does not alter the pool of release-ready presynaptic vesicles and therefore could not cause the observed reduced basal neurotransmission in animals with reduced postsynaptic sensitivity. However, we found that presynaptic active zone scaffold, Bruchpilot (Brp), undergoes a rapid synaptic accumulation upon pharmacological induction of PHP in control animals but not in neto-α\textsuperscript{null} mutants (Figure 3). The findings suggest that, in response to reduced postsynaptic sensitivity, Neto-α functions to swiftly mobilize Brp, which presumably enhances vesicle release and enables the compensatory response.

Our data demonstrate that the two major functions of Neto-α in the presynaptic compartment could be segregated and mapped to different domains: (1) the minimal Neto, or Neto-deltaCTD, which modulates basal neurotransmission, likely by modulating the KaiRID function; and (2) the intracellular part of Neto-α, which is both required and sufficient for the presynaptic homeostatic response. The limiting Neto-α appears to be recruited at synapses by the presynaptic KaiRID, a finding that challenges our current thinking that auxiliary subunits “assist” iGluRs and that provides an exquisite example of an auxiliary protein that performs a key synaptic function with assistance from iGluRs.

**Differential subcellular distribution for Neto isoforms**

Intriguingly, neuronal expression of Neto-β cannot rescue the basal neurotransmission and PHP deficits at neto-α\textsuperscript{null} NMJs, which is unexpected, because Neto-β contains all the domains of the “minimal Neto” (Neto-deltaCTD) and, at the least, should have rescued the basal neurotransmission levels. We examined the Neto-β–specific signals and found that neuronal overexpression of Neto-β did not induce accumulation along axons or at synaptic terminals; instead, Neto-β remained restricted to the somato-dendritic compartment. Current efforts focus on elucidating the protein motifs and the molecular pathways that block the trafficking of Neto-β through the axonal initiation segment and into the axonal compartment and restrict Neto-β to the somato-dendritic compartment.

Together, our studies provide strong evidence for Neto activities at presynaptic terminals. Previous work showed that presynaptic KARs regulate neurotransmitter release; however, the site and mechanism of action of presynaptic KARs have been difficult to pin down. Our structure-function analyses, together with the fact acute PHP induction occurs even when the axon is severed, indicate that Neto-α together with KaiRID, localizes at presynaptic terminals, where KaiRID could function as an auto-receptor.

Our current efforts focus on identifying proteins that interact with Neto both inside and outside the cell and provide critical activities at the developing NMJ, including: (1) iGluR-clustering; (2) iGluR recruitment and stabilization at PSDs; and (3) mediating PHP. To this end, we initiated several complementary screens: pull-down and mass-spectroscopy comparisons of proteins interacting with the intracellular domains of the fly Neto proteins (α and β); and a synthetic lethality screen.
Local BMP/BMPR complexes regulate synaptic plasticity.

Synaptic activity and synapse development are intimately linked, but our understanding of the coupling mechanisms is limited. In particular, how synapse activity status is monitored and communicated across the synaptic cleft remains poorly understood. Our previous studies uncovered a role for bone morphogenetic proteins (BMPs) in sensing the activity of post-synaptic receptors and relaying this information across the synaptic cleft.

At the *Drosophila* NMJ, BMP signaling is critical for NMJ growth, neurotransmitter release, and synapse plasticity and homeostasis. We discovered a completely novel BMP signaling modality that operates in conjunction with the canonical BMP pathway to ensure those functions. The canonical BMP pathway, triggered by muscle-derived Gbb binding to presynaptic BMP type-II receptor (BMPRII), Wishful thinking (Wit), and the BMPRIs Thickveins (Tkv) and Saxophone (Sax), induces accumulation of phosphorylated Smad (pMad) in motor-neuron nuclei and activates transcriptional programs with distinct roles in the structural and functional development of the NMJ. Gbb and Wit also signal non-canonically through the effector protein LIM kinase 1 (LIMK1) to regulate synapse stability. Interestingly, pMad also accumulates at synaptic locations, but the biological relevance of the phenomenon remained a mystery for over a decade. We found that synaptic pMad constitutes a sensor for synaptic activity. Furthermore, we previously found that synaptic pMad marks a novel, noncanonical BMP pathway, genetically distinguishable from all other known BMP signaling cascades. This novel pathway stabilizes postsynaptic type-A (GluRIIA–containing) receptors as a function of their activity.

Type-A receptors are the first to arrive at a nascent synapse; they form the “core” of the receptor field and are surrounded by type-B receptors. Previous work in our and other labs established that the incorporation of type-A receptors in stable synaptic complexes depends on GluRIIA activity and requires an extensive postsynaptic network. Our studies on the novel BMP signaling modality indicate that the synaptic stabilization of type-A receptors also requires trans-synaptic complexes. The question arises as to how postsynaptic glutamate receptors modulate presynaptic pMad and are in turn stabilized by it. Given that synaptic pMad depends on active type-A receptors, we favor a model whereby Neto, via its BMP–binding CUB domains, connects active postsynaptic type-A receptors with presynaptic BMP/BMPR complexes (Figure 4). Such trans-synaptic complexes could offer a versatile means for relaying synapse activity status to the presynaptic neuron via fast conformational modifications. At the same time, the trans-synaptic complexes may facilitate interactions that stabilize the type-A receptors at PSDs, a positive feedback that could explain the Hebbian mode of GluRIIA incorporation at the PSD and maturation of iGluR fields at larval NMJs. Given that BMPRs are...
limiting and shared among different BMP signaling modalities, neurons may use this novel BMP pathway to monitor synapse activity and then coordinate NMJ growth with synapse maturation and stabilization.

**Selective disruption of synaptic BMP signaling by a Smad mutation adjacent to the conserved H2 helix**

In search of Mad features that influence its association with the BMPRs, we collected most of existing *Drosophila* Mad alleles and compared them for their ability to sustain the two Smad–dependent signaling modalities: canonical BMP signaling, marked by pMad accumulation in motor neuron nuclei, and Smad–dependent local, synaptic signaling, marked by pMad accumulation at synaptic terminals. Within this comprehensive collection, we found that strong Mad alleles generally disrupt both synaptic and nuclear pMad accumulation, whereas moderate Mad alleles have a wider range of phenotypes and selectively impact different BMP signaling modalities. Interestingly, regulatory Mad mutations reveal that synaptic pMad appears to be more sensitive to a net reduction in Mad levels than is nuclear pMad. Importantly, a previously uncharacterized allele, Mad<sup>8</sup> showed markedly reduced synaptic pMad levels but only moderately diminished nuclear pMad. The postsynaptic composition and electrophysiological properties of Mad<sup>8</sup> NMJs were likewise altered. Using biochemical assays and structural modeling, we examined how point mutations such as S359L (marked*, Figure 5), present in Mad<sup>8</sup>, could influence the Mad-receptor interface, and we identified a key motif, the H2 helix [Reference 2]. Our study highlights the biological relevance of Smad–dependent, synaptic BMP signaling and uncovers a highly conserved structural feature of Smads that is critical for normal development and function.
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Publications


Collaborators

• Mary Dasso, PhD, *Section on Cell Cycle Regulation, NICHD, Bethesda, MD*

• Gregory T. Macleod, PhD, *Florida Atlantic University, Jupiter, FL*

• Mark Mayer, PhD, *Scientist Emeritus, NINDS, Bethesda, MD*

• Stuart Newfeld, PhD, *School of Life Sciences, Arizona State University, Tempe, AZ*

Contact

For more information, email *mihaela.serpe@nih.gov* or visit *http://ucc.nichd.nih.gov*. 
Thyroid Hormone Regulation of Vertebrate Postembryonic Development

The laboratory investigates the molecular mechanisms of thyroid hormone (TH) function during postembryonic development, a period around mammalian birth when plasma TH levels peak. The main model is the metamorphosis of *Xenopus laevis* and *X. tropicalis*, two highly related species, which offer unique but complementary advantages. The control of this developmental process by TH offers a paradigm in which to study gene function in postembryonic organ development. During metamorphosis, different organs undergo vastly different changes. Some, like the tail, undergo complete resorption, while others, such as the limb, are developed *de novo*. The majority of larval organs persist through metamorphosis but are dramatically remodeled to function in a frog. For example, tadpole intestine is a simple tubular structure consisting primarily of a single layer of larval epithelial cells. During metamorphosis, it is transformed, through specific larval epithelial cell death and *de novo* development of the adult epithelial stem cells, followed by their proliferation and differentiation, into an organ with a multiply folded adult epithelium surrounded by elaborate connective tissue and muscles. The wealth of knowledge from past research and the ability to manipulate amphibian metamorphosis, both *in vivo* by using genetic approaches or hormone treatment of whole animals, and *in vitro* in organ cultures, offer an excellent opportunity firstly to study the developmental function of TH receptors (TRs) and the underlying mechanisms *in vivo* and, secondly, to identify and functionally characterize genes that are critical for organogenesis, particularly for the formation of the adult intestinal epithelial stem cells during postembryonic development in vertebrates. A major recent focus has been to make use of the TALEN and CRISPR/Cas9 technologies to knockdown or knockout the endogenous genes for functional analyses. In addition, the recent improvements in *Xenopus tropicalis* genome annotation allow us to carry out RNA-Seq and chromatin-immunoprecipitation (ChIP)-Seq analyses at the genome-wide level. We complement our frog studies by investigating the genes found to be important for frog intestinal stem-cell development in developing mouse intestine by making use of the ability to carry out conditional gene knockout.

**Identification of notochord-enriched genes induced during *Xenopus tropicalis* tail resorption**

Tail resorption during anuran metamorphosis is perhaps the most dramatic tissue transformation to occur during vertebrate development.
FIGURE 1. Schematics showing the effects of TRα (thyroid hormone receptor α) knockout on *Xenopus tropicalis* development

TRα knockout has little effect on embryogenesis, and resulting tadpoles are normal by feeding stage (stage 45). However, once feeding begins, the animals grow at different rates, with the knockouts growing faster; they are thus larger than wild-type siblings at the same age (in days) (comparing the vertical axis values of the lines for the knockout and wild-type animals at any given position along the horizontal axis between stages 45 and 54). The knockout animals also develop faster, reaching developmentally more advanced stages than wild-type siblings at the same age (in days). Thus, the knockout animals reach stage 54, the onset of metamorphosis, at a younger age (see the horizontal axis locations for the upper end of the lines). Interestingly, when the animals are compared at stage 54, the wild-type are larger than the knockout siblings, even though the latter grow faster. This is because the wild-type animals take longer to reach metamorphosis (stage 54). The extra growth time needed to reach stage 54 enables the wild-types to catch up and surpass the knockouts in size. After the initiation of metamorphosis at stage 54, the knockout tadpoles metamorphose more slowly than the wild-types, enabling the latter to catch up in development, with both groups and finishing metamorphosis at around the same age. The knockout animals initiate metamorphosis at a smaller size and also end up smaller at the end of metamorphosis than do the wild-type siblings. Thus, in metamorphic tadpoles prior to stage 54, unliganded TRα (due to the lack of thyroid hormone) functions to control metamorphic timing, whereas, when thyroid hormone becomes available during metamorphosis, TRα helps increase the rate of metamorphosis.

Like all other processes during metamorphosis, tail resorption is controlled by TH. Earlier studies in the highly related anuran species *Xenopus laevis* and *Xenopus tropicalis* showed that TR plays a necessary and essential role in metamorphosis. Of the two known TR genes in all vertebrates, *tra* is highly expressed during both premetamorphosis and metamorphosis, while *trβ* expression is low in premetamorphic tadpoles but highly upregulated as a direct target gene of TH during metamorphosis, especially in the tail. Gene knockout studies showed that *trβ* knockout significantly delays late metamorphosis, particularly tail resorption, resulting in tailed frogs well after wild-type siblings complete metamorphosis. Most noticeably, in *trβ*-knockout tadpoles, an apparently normal notochord is present in the tail as late as three days after the initiation of tail shortening (stage 62), while in wild-type and *tra*-knockout tadpoles, the tail notochord disappears in about one day. The findings suggest the existence of TRβ-regulated notochord-specific gene expression program during tail resorption.

We carried out a comprehensive gene expression analysis in the notochord during metamorphosis using RNA-Seq analyses of whole tail at stage 60 before any noticeable tail length reduction, whole tail at stage 63 when the tail length is reduced by about one half, and the rest of the tail at stage 63 after removing the notochord. This allowed us to identify many notochord-enriched, metamorphosis-induced genes at stage 63 [Reference1]. Future studies on these genes should help determine whether they are regulated by TRβ and play any roles in notochord regression. We also discovered differential regulation of several matrix metalloproteinases (MMPs), which are
known to be upregulated by TH and thought to play a role in tissue resorption by degrading the extracellular matrix (ECM). In particular, MMP9-TH and MMP13 are extremely highly expressed in the notochord compared with the rest of the tail. In situ hybridization analyses showed that these MMPs are expressed in the outer sheath cells and/or the connective tissue sheath surrounding the notochord. Our findings suggest that high levels of trβ expression in the notochord specifically upregulate the MMPs, which in turn degrades the ECM, leading to the collapse of the notochord and its subsequent resorption during metamorphosis.

Organ-specific requirements for thyroid hormone receptor ensure temporal coordination of tissue-specific transformations and completion of Xenopus metamorphosis.

We generated tra and trβ double knockout animals and carried out molecular and morphological analyses to determine whether TR is required for Xenopus development [Reference 2]. We found that the tr double knockout tadpoles do not respond to TH, supporting the view that there are no other TR genes in Xenopus tropicalis and that TR is essential for mediating the effects of TH in vivo. Surprisingly, the double knockout tadpoles are able to initiate metamorphosis and accomplish many metamorphic changes, such as limb development. However, all double knockout tadpoles stall and eventually die at stage 61, the climax of metamorphosis, before tail resorption takes place. Analyses of the knockout tadpoles at stage 61 revealed various developmental abnormalities, including precocious ossification and extra vertebrae. Our data indicate that TRs are not required for the initiation of metamorphosis but are essential for its completion. Furthermore, the differential effects of tr knockout on different organs/tissues suggest tissue-specific roles for TR to control temporal coordination and progression of metamorphosis in various organs.

Function of the TR-coactivator histone methyltransferase PRMT1 during Xenopus tropicalis development

Asymmetric arginine dimethylation of histone H4R3 to H4R3me2a by protein arginine methyltransferase 1 (PRMT1), a TR-coactivator that is also upregulated by TH during metamorphosis, is thought to play a key role in gene activation throughout vertebrates. PRMT1 knockout in mouse leads to embryonic lethality. This and the uterus-enclosed nature of the mouse embryo make it difficult to determine the role of PRMT1 in mammal development. We took advantage of the external development of the diploid anuran Xenopus tropicalis and adapted the TALEN genome editing technology to knock out PRMT1 in order to investigate how PRMT1 participates in vertebrate development [Reference 3]. We observed that PRMT1 knockout had no apparent effect on embryogenesis because normally feeding tadpoles were formed, despite the reduced asymmetric H4R3 di-methylation (H4R3me2a) resulting from the knockout. However, PRMT1 knockout tadpoles exhibited severely reduced growth even with normal growth-hormone gene expression. The development of such tadpoles was also stalled shortly after feeding began at stages 44/45, and they died within two weeks, well before the onset of metamorphosis. In situ analyses revealed broad cessation of or drastic reduction in cell proliferation in diverse organs including the eye, brain, spinal cord, liver, and intestine. Our findings suggest that PRMT1 is not required for embryogenesis but is a key regulator in the normal progression of vertebrate development and growth.

TRb is critical for intestinal remodeling during Xenopus tropicalis metamorphosis.

We analyzed the effect of trβ knockout on TH–induced intestinal remodeling using animals containing an out-frame-mutation of a five-base deletion generated with the CRISPR/Cas9 gene-editing technology [Reference 4].
We observed that trβ knockout does not affect premetamorphic tadpole development. However, we found that the basal expression of direct TH–inducible genes is increased and their upregulation by TH is reduced in the intestine of premetamorphic homozygous trβ knockout animals and is accompanied by reduced target binding by TR. More importantly, we observed reduced adult stem cell proliferation and larval epithelial apoptosis in the intestine during TH–induced metamorphosis. Our data suggest that TRβ plays a critical role in intestinal remodeling during metamorphosis.

The tRNA methyltransferase–like 1 gene (Mettl1) is directly regulated by thyroid hormone receptor during Xenopus tropicalis metamorphosis, implicating a role in adult intestinal stem-cell development and proliferation.

We previously used ChIP-on-chip assays to identify many putative TR target genes, among them the tRNA methyltransferase Mettl1. We studied the regulation of the mettl1 gene by TH during intestinal metamorphosis, a process that involves near complete degeneration of the larval epithelial cells via apoptosis and de novo formation of adult epithelial stem cells and their subsequent proliferation and differentiation. We observed

FIGURE 2. Intestinal metamorphosis involves the formation of clusters of proliferating, undifferentiated epithelial cells at the climax.

Tadpoles at premetamorphic stage 54 (A), climax (B, stage 62), and end of metamorphosis (C, stage 66) were injected with 5-ethynyl-2′-deoxyuridine (EdU) one hour before being sacrificed. Cross-sections of the intestine from the resulting tadpoles were double-stained by EdU labeling of newly synthesized DNA and by immunohistochemistry of IFABP (intestinal fatty acid–binding protein), a marker for differentiated epithelial cells. The dotted lines depict the epithelium-mesenchyme boundary. Note that there are few EdU–labeled proliferating cells in the epithelium and that they express IFABP at premetamorphosis (A) and increase in the form of clustered cells (proliferating adult stem cells), which lack IFABP at the climax of metamorphosis (B). At the end of metamorphosis, EdU–labeled proliferating cells are localized mainly in the troughs of the epithelial folds, where IFABP expression is low (C). ep, epithelium; ct, connective tissue; m, muscles; l, lumen.
that mettl1 was activated by TH in the intestine during both natural and TH-induced metamorphosis and that its mRNA level peaks at the climax of intestinal remodeling. We further showed that the mettl1 promoter could be activated by liganded TR via a TH response element (TRE) upstream of the transcription start site in vivo. More importantly, we found that TR binding to the TRE region correlated with the increase in the level of H3K79 methylation, a transcription-activation histone mark, and the recruitment of RNA polymerase II by TH during metamorphosis. Our findings suggest that, in the intestine during metamorphosis, mettl1 is activated by liganded TR directly at the transcriptional level via the TRE in the promoter region. Mettl1 in turn regulates target tRNAs to affect translation, thus facilitating stem-cell formation and/or proliferation during intestinal remodeling.

**Wnt promotes amino-acid transporter LAT1 to constrain the integrated stress response during mouse embryogenesis.**

To regulate cellular processes, TH has to be actively transported into cells, a process that is mediated by several different types of transporters. One of our previously identified TH–response genes in the intestine, lat1, encodes the light chain of a heterodimeric system L type of TH transporter, which also transports several amino acids. Interestingly, lat1 is highly upregulated at the climax of metamorphosis in the tadpole intestine, coinciding with the formation and rapid proliferation of the adult intestinal stem cells. In addition, we found that Lat1 was also highly expressed in the mouse intestine during the neonatal period when the mouse intestine matures into the adult form, a process that appears also involves TH–dependent formation and/or proliferation of the adult intestinal stem cells. Through collaborative studies, we generated a mouse line with the Lat1 gene floxed, which allows conditional knockout of the Lat1v upon expression of the Cre recombinase. More recently, we showed that Lat1 is highly expressed in mouse tissues undergoing morphogenesis and that Lat1–null mouse embryos, generated by crossing the Lat1–floxed mice with a mouse line expressing Cre ubiquitously, have profound neural and limb-bud outgrowth defects [Reference 5]. Lat1–null neural tissue exhibited mild proliferation defects and aberrant mTORC1 activity; transcriptomics and protein phosphorylation and apoptosis analyses further indicated that induction of the integrated stress response is the likely cause of the observed defects. We also detected the pattern of stress-response gene expression induced in Lat1–null embryos at a low-level in wild-type embryos and identified stress-vulnerability specifically in tissues undergoing morphogenesis. The Lat1–null phenotype is reminiscent of Wnt–pathway mutants, and we showed that loss of Wnt or β-catenin inhibits Lat1 expression and induces the stress response. Wnt signaling therefore normally supports the metabolic demands of morphogenesis and constrains cellular stress. Moreover, operation of the integrated stress response in the embryo, which is pathogen-mediated as well as triggered by metabolic stress, may provide a mechanistic explanation for a range of developmental defects.

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**Publications**


**Collaborators**

- Sheue-Yann Cheng, PhD, *Laboratory of Molecular Biology, NCI, Bethesda, MD*
- Steven Coon, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Eiichi Hinoi, PhD, *Kanazawa University Graduate School, Kanazawa, Japan*
- James Iben, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Jianping Jiang, PhD, *Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China*
- Tianwei Li, PhD, *Molecular Genomics Core, NICHD, Bethesda, MD*
- Keisuke Nakajima, PhD, *Amphibian Research Center, Hiroshima University, Hiroshima, Japan*
- Bingyin Shi, MD, *Xi’an Jiaotong University School of Medicine, Xi’an, China*
- Kate G. Storey, PhD, *University of Dundee, Dundee, UK*
- Guihong Sun, PhD, *Wuhan University School of Medicine, Wuhan, China*
- Peter Taylor, PhD, *University of Dundee, Dundee, UK*
- Henry Zhang, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*

**Contact**

For more information, email shi@helix.nih.gov or visit http://smm.nichd.nih.gov.
Modeling the Biophysics of the Membrane

The integrity of lipid membranes is essential for life. They provide spatial separation of the chemical contents of the cell and thus make possible the electrical and chemical potential differences that are used to transmit signals and perform work. However, the membrane must be broken frequently to form, for example, new membrane structures in the cell. The simplest structure is a vesicle to transport cargo. Such vesicles are constantly cycled between organelles and the outer plasma membrane. Thus, there is a careful balance between boundary-establishing membrane fidelity and the necessary ability of the cell to change these boundaries.

The challenge in studying the membrane is its complexity. The membrane is a thin sheet of small molecules, i.e., lipids. There are hundreds of types of lipids in the cell. Each lipid changes the properties of the membrane in its vicinity, sometimes making the sheet stiffer, sometimes softer, and sometimes acting to bend the membrane into a ball or tube. Furthermore, the lipids are constantly jostling and tangling with both each other and with proteins embedded in the membrane. To predict of how membranes are reshaped thus requires not only knowing how lipids affect the properties of the membrane surface, but also the location of specific lipids.

The question as to how molecular scale features influence extensive biological processes must be answered in the language of physical laws. Physics is the language of mechanism at the molecular scale. The challenge is linking physics to the “big” processes that happen in life. Our lab uses detailed physics-driven molecular simulation to ‘build up’ models that can be applied at the much larger level of the cell, which requires retaining important information and eliminating irrelevant details. The software our lab develops is based on the models that we are building. Thus, a broad objective of our research is to create a publicly available software package that can be used either as a stand-alone application for analyzing membrane-reshaping processes or as a library for cellular-scale modeling packages for which the role of the membrane may be unclear or unanticipated.

Another key component of our research is to seek the best possible validation of our models. Few techniques are able to yield molecular information about lipids. Recent breakthroughs that break the
Determining the structure of complex membranes with neutron scattering and molecular simulation

The structure of molecular bilayers is challenging to characterize owing to its dynamic nature. Structural biological techniques can resolve the atomic positions of proteins because a protein is represented well by a single or limited number of structures. In contrast, biological membranes are highly dynamic, meaning both that the surface as a whole undulates (like the surface of a lake) and that the many constituent lipids rearrange rapidly into different patterns determined by their chemistry. The patterns are undetectable by the methodology used to characterize proteins.

Neutrons offers exciting advantages over light microscopy. Unlike a fluorophore (or dye) that absorbs and emits (inelastically) photons from its diffuse electronic cloud, neutrons interact elastically with the atomic nuclei of lipids. That is, if one shoots a beam of neutrons at a sample, they bounce off the atoms of the sample, changing direction but not speed. At this quantum length-scale, they generate a specific pattern at the detector of a neutron experiment. While this so-called interference pattern reflects that complex patterning of lipids on a surface, it is nearly impossible to directly reconstruct into the original shape and patterning of lipids. With our new developments, scientists can use molecular simulations as a practical bridge between signal and structure.

In two studies we published this year, we develop new approaches that enable molecular simulations to capture the scattering from experimental samples with complex lipid compositions. The first, in collaboration with Fred Heberle, Edward Lyman, and John Katsaras, computes “lateral” scattering from bilayers. The majority of scattering from a bilayer reflects its shape as a thin sheet; this characteristic signal has been previously well characterized. A much smaller component of the scattering is a result of the in-plane lateral distributions of lipids. Understanding the component is critically important for determining how lipid chemistry leads to rearrangement within the surface, and eventually what shapes a bilayer will form. Developing this method, along with the accompanying software, required significant mathematical developments, as well as an extensive validation test [Reference 1].

We also modelled the scattering from large, highly curved bilayer structures, such as vesicles. The methodology [Reference 2] combines our continuum and molecular simulations to create a complete picture of scattering from complex objects. Essentially, one can now conduct a relatively short simulation of a complex lipid bilayer followed by a computationally modest continuum model of a large shape (too large for a molecular simulation), and compute the full neutron scattering.
**Publications**


**Collaborators**

- Frederick Heberle, PhD, *Oak Ridge National Laboratory, Oak Ridge, TN*
- John Katsaras, PhD, *Oak Ridge National Laboratory, Oak Ridge, TN*
- Edward Lyman, PhD, *University of Delaware, Newark, DE*
- Lutz Maibaum, PhD, *University of Washington, Seattle, WA*

**Contact**

For more information, email [alexander.sodt@nih.gov](mailto:alexander.sodt@nih.gov) or visit [https://www.nichd.nih.gov/research/atNICHD/Investigators/sodt](https://www.nichd.nih.gov/research/atNICHD/Investigators/sodt).
The main goal of the research in our Section is to examine cell-signaling cascades, gene expression, and hormone secretion in neuroendocrine cells from the hypothalamus and pituitary gland during development. We place special emphasis on characterization of individual cells, using fluorescence imaging, patch-clamp recordings, simultaneous membrane potential/calcium and current/calcium recordings, electrophysiological and imaging recordings of single-cell exocytic events, single-cell RNA sequencing (scRNASeq), and single-cell quantitative reverse transcription polymerase chain reaction (RT-PCR). Our recent and ongoing work has focused on signaling, transcription, and secretion in the pituitary gland specific for age, sex, and tissue structure; pituitary cell heterogeneity, reflecting their postnatal gene expression; the role of 1-phosphatidylinositol 4-kinases and protein receptor tyrosine N2 phosphatase type in postnatal proliferation and maintenance of pituitary lineages; as well as cell-specific electrical activity and exocytic pathways. Current and proposed studies depend in part on the use of equipment in NICHD's Microscopy and Imaging Core Facility and the Molecular Genomics Core Facility.

**Enhancement of analysis of scRNASeq data: pituitary cell example**

The scRNASeq investigation of the pituitary required the development or adaptation of methods for data analysis. The main problem in the analysis was related to the fact that several highly expressed transcripts, including those of *Gh1*, *Prl*, *Pomc*, *Lhb*, and *Tshb*, also showed the presence at lower levels in cell types known not to have expressed them, for example *Gh1* in erythrocytes and *Hbb* in somatotrophs. To clarify the nature of this background, we added HEK293 cells expressing a GFP–tagged protein to pituitary glands, dispersed the cells, and examined *Gh1* expression by single-cell qRT–PCR (quantitative RT-PCR). The analysis showed that all HEK293 cells from such a preparation, but not the control cells, were *Gh1*–positive, indicating that RNA released from broken cells during dispersion binds extracellularly to neighboring cells, resulting in contamination. To overcome the problem, we developed a threshold calculation method for each gene based on the Otsu’s method to define whether the gene is expressed above background levels. Combined with standard quality-control techniques, such as the removal of potentially...
damaged cells based on a high proportion of mitochondrial transcripts, the threshold allowed us to develop a logic-based cell-type classification method using marker genes. We also developed a pituitary-specific clustering program for scRNASeq, based on integration of the major cell type–specific genes, along with a method for identification of marker and dominant genes, and separating proliferative from differentiated cells. Such methodological achievements will be highly useful in our current and future work with scRNASeq analysis of rat and human pituitary cells, where the separation of undifferentiated, proliferative, and differentiated cell types, including the presence of bihormonal cells, is essential [Reference 1].

Dependence of gonadotroph marker-gene expression on GnRH application patterns and animal immune status

Mammalian reproduction depends on the proper synthesis and release of two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), by specialized anterior pituitary endocrine cells called gonadotrophs. The hormones are dimeric glycoproteins composed of a common glycoprotein hormone, α polypeptide (Cga), and unique β subunits (Lhb and Fshb), which give biological specificity. Gonadotropin secretion is tightly regulated by hypothalamic and intrapituitary factors. Among them, the most important is gonadotropin-releasing hormone (GnRH), which is released in a pulsatile manner by a small set of neurons within the preoptic area and the mediobasal hypothalamus. Upon reaching the anterior pituitary, GnRH binds to its receptor expressed in gonadotrophs and signals through a Gq/11–dependent cascade (Gq/11 is a heterotrimeric guanine nucleotide [G]–binding protein) of intracellular pathways that culminate in periodic gonadotropin secretion. The pulsatile pattern of GnRH release is crucial for proper gonadotropin synthesis and release. Several physiological responses and pathological conditions that cause reproductive failure in humans have been associated with impaired regulation of pulsatile GnRH release, including functional hypothalamic amenorrhea, hyperprolactinemia, polycystic ovary syndrome, and hypogonadotropic hypogonadism. Continuous treatment with a GnRH receptor agonist is also clinically relevant, for example, for the prevention of ovarian hyperstimulation syndrome during assisted reproduction, for ovarian protection during chemotherapy, and for the treatment of precocious puberty and polycystic ovary syndrome.

We recently assessed the time course of expression of the gonadotroph marker genes Fshb, Lhb, Gnrhr, and Cga in static cultures of rat anterior pituitary during continuous treatment of GnRH or its analogs. We also studied the expression of the transcription factor genes Egr1, Nr5a1, and Pitx1, which are critical for the control of Lhb expression. In addition, we characterized the acute and long-term effects of cell dispersion and primary culture in the absence of GnRH for these genes and commonly used reference genes, before examining the effect of continuous GnRH treatment. Further, we studied the relationship between Lhb expression, LH cell content, and LH release in vitro during continuous GnRH treatment. We also evaluated the effects of in vivo injection of a GnRH–receptor agonist and antagonist on gonadotropin subunit gene expression and serum and pituitary LH content. We used the expression of two SIBLING genes (a subfamily of the secreted calcium-binding phosphoproteins), Dmp1 and Spp1, which we recently characterized, as internal controls for in vitro and in vivo experiments.

Culturing of pituitary cells in GnRH–free conditions reduced Fshb, Cga, and Gnrhr expression, while continuous treatment with GnRH agonists upregulated Cga expression progressively and Gnrhr and Fshb expression transiently, which was accompanied by a prolonged blockade of Fshb but not Gnrhr expression. In contrast, Lhb expression was relatively insensitive to the loss of endogenous GnRH and continuous treatment with GnRH, probably reflecting the expression status of Egr1 and Nr5a1. We observed similar response patterns in vivo.
FIGURE 1. Effects of a drop in extracellular pH on current responses in immortalized GH3 cells

A–D. Patterns of proton-activated currents in voltage-clamped single cells: a biphasic response composed of a transient spike phase and a sustained small amplitude plateau phase during long-term stimulation with pH 6.3 (A and B), and two-current responses to pH 4.5 (C and D) in different cells.

E and F. Proton concentration dependence of the peak amplitudes of fast and slow current responses; representative traces from a single cell during repeated proton application (E) and mean ± SEM values derived from 5–10 single-cell recordings per dose (F). Traces shown are representative of 6 to 10 cells per experiment.

after administration of GnRH agonists. However, continuous treatment with GnRH stimulated LH secretion in vitro and in vivo, leading to a reduction in LH cell content despite high basal Lhb expression. The data suggest that blockade of Fshb expression and depletion of the LH secretory pool are two major factors accounting for weakening of the gonadotroph secretory function during continuous GnRH treatment [Reference 2].

Multiple sclerosis develops in a sex-specific manner during reproductive years. Various neuroendocrine changes in this inflammatory, demyelinating, and debilitating disease have been described. Recently, we aimed to determine the extent and gender specificity of changes in the hypothalamic-pituitary-gonadal axis called experimental autoimmune encephalomyelitis in a rat multiple-sclerosis model. During the course of the disease, hypothalamic tissue showed transient upregulation of the inflammatory marker genes Gfap, Cd68, Ccl2, and Il1b in both sexes, but was accompanied by sex-specific decrease in Kiss1 (in females only) and Gnrh1 (in males only) expression. In females, the expression of the gonadotroph-specific genes Lhb, Cga, and Gnrhr was also inhibited, accompanied by decreased basal but not stimulated serum luteinizing hormone levels and temporary arrest of the estrous cycle. In contrast, Fshb expression and serum progesterone levels were temporarily elevated, findings consistent with the maintenance of the corpora lutea, and increased immunohistochemical labeling of ovarian StAR (steroidogenic acute regulatory protein), a protein that limits the rate of the steroidogenic pathway. In males, decreased regulation of Gnrhr expression and basal and stimulated serum LH and testosterone levels were accompanied by inhibited expression of the StAR protein in the testis. We suggest that inflammation of the hypothalamic tissue reduces the expression of Kiss1 and Gnrh1 in females and males, respectively, leading to sex-specific changes downstream of the axis [Reference 3].

Cell type–specific expression pattern of proton-sensing receptors and channels in pituitary gland

Extracellular protons act as orthosteric ligands for proton-sensing G protein–coupled receptors (GPRs) and acid-sensing ion channels (ASICs), as well as allosteric regulators of other GPRs and channels. Four orphan
GPRs, GPR4, GPR65, GPR68, and GPR132, sense extracellular protons through histidine residues of the receptors and signal through heterotrimeric G-proteins, including $G_s$, $G_i$, $G_q$, and $G_{12/13}$, triggering different intracellular signaling pathways. ASICs are a class of voltage-independent proton-gated sodium-conducting receptor channels. There are seven subunits (ASIC1a, ASIC1b, ASIC2a, ASIC2b, ASIC3, ASIC4, and ASIC5) encoded by five genes. All subunits are composed of two transmembrane domains, a large extracellular loop, and cytoplasmic N- and C-termini. Functional channels consist of three subunits in a homomeric or heteromeric configuration. The expression of these receptors and channels and their roles in pituitary-cell functions have not been systematically investigated.

We recently completed a study on the cell type–specific expression pattern of proton-sensing GPRs and ASICs in rat anterior pituitary cells and GH3 immortalized lactosomatotrophs (GH3 cells). Our work includes scRNASeq, RT-PCR, and qRT-PCR analyses, double immunostaining, and single-cell electrophysiological analysis. qRT-PCR analysis revealed expression of the G protein–coupled receptor 68 gene ($\text{Gpr68}$) and the ASIC genes $\text{Asic1}$, $\text{Asic2}$, and $\text{Asic4}$ in anterior pituitary cells, and $\text{Asic1}$ and $\text{Asic2}$ in immortalized GH3 pituitary cells. $\text{Asic1a}$ and $\text{Asic2b}$ were the dominant splice isoforms. Single anterior pituitary cell RNA sequencing and immunocytochemical analysis showed that non-excitatory folliculostellate cells express the $\text{Gpr68}$ gene and protein, while excitable secretory cells express ASIC genes/proteins. $\text{Asic1}$ was detected in all secretory cell types, $\text{Asic2}$ in gonadotrophs, thyrotrophs, and somatotrophs, and $\text{Asic4}$ in lactotrophs only. Extracellular acidification activated two types of currents in a concentration-dependent manner: a rapidly developing, desensitizing current, with an estimated EC50 value of pH 6.7; and a slowly developing, non-desensitizing current, which required a higher proton concentration for activation. The desensitizing current was eliminated by removal of bath sodium and application of amiloride, a blocker of ASIC channels, whereas the non-desensitizing current was amiloride-insensitive and voltage-dependent. Activation of both currents increased the excitability of secretory pituitary cells, consistent with their potential physiological relevance in the control of voltage-gated calcium influx and calcium-dependent cellular functions [Reference 4].
Expression and role of TSH receptors in proopiomelanocortin-producing pituitary cells

It is well established that thyroid-stimulating hormone (TSH) regulates thyroid hormone synthesis and release from the thyroid gland by activating TSH receptors expressed in thyroid follicular cells. In the human thyroid gland, TSH receptors not only interact with $G_s$ and $G_{q/11}$ proteins (G protein coupled receptors), leading to adenylyl cyclase and phospholipase C activation, respectively, but are also capable of signaling though $G_{i/o}$ and $G_{12/13}$ proteins [Reference 4]. Several extra-thyroidal expression sites of TSH receptors have also been reported [Reference 5], including in normal and adenomatous human pituitary tissue. However, the cell type(s) expressing the receptors have not been identified.

We recently examined whether the functional TSH receptors are also expressed in cultured rat pituitary cells, using double immunocytochemistry, qRT-PCR analysis, CAMP and hormone measurements, and single-cell calcium imaging. Double immunocytochemistry revealed the expression of TSH receptors in cultured corticotrophs and melanotrophs, in addition to previously identified receptors in folliculostellate cells. Functional coupling of the receptors with the $G_{q/11}$–signaling pathway was not observed, as evidenced by the lack of TSH activation of inositol trisphosphate (IP3)–dependent calcium mobilization in the cells when bathed in calcium-deficient medium. However, TSH increased cAMP production in a time- and concentration-dependent manner and facilitated calcium influx in single corticotrophs and melanotrophs, indicating their coupling to the $G_s$–signaling pathway. Consistent with these findings, TSH stimulated adrenocorticotropin and beta-endorphin release in male and female pituitary cells in a time- and concentration-dependent manner without affecting expression of the proopiomelanocortin gene. This ongoing research indicates that TSH is a potential paracrine modulator of anterior pituitary corticotrophs and melanotrophs, controlling the exocytotic but not the transcriptional pathway in a cAMP/calcium influx–dependent manner.

Publications


Collaborators

• Ivana Bjelobaba, PhD, *University of Belgrade, Beograd, Serbia*
• Patrick A. Fletcher, PhD, *Laboratory of Biological Modeling, NIDDK, Bethesda, MD*
• Arthur Sherman, PhD, *Laboratory of Biological Modeling, NIDDK, Bethesda, MD*

Contact

For more information, email *stojilks@mail.nih.gov* or visit [https://www.nichd.nih.gov/research/atNICHD/Investigators/stojilkovic](https://www.nichd.nih.gov/research/atNICHD/Investigators/stojilkovic) or [https://dir.ninds.nih.gov/Faculty/Profile/stanko-stojilkovic.html](https://dir.ninds.nih.gov/Faculty/Profile/stanko-stojilkovic.html).
Response heterogeneity and adaptation in olfactory receptor neurons

The olfactory system, consisting of relatively few layers of neurons, with structures and mechanisms that appear repeatedly in widely divergent species, provides unique advantages for the analysis of information processing by neurons. Olfaction begins when odorants bind to olfactory receptor neurons, triggering them to fire patterns of action potentials. Recently, using new electrophysiological recording tools, we found that the spiking responses of olfactory receptor neurons are surprisingly diverse and include powerful and variable history dependencies. Single, lengthy odor pulses elicit patterns of excitation and inhibition that cluster into four basic types. Different response types undergo different forms of adaptation during lengthy or repeated stimuli. A computational analysis showed that such diversity of odor-elicited spiking patterns helps the olfactory system efficiently encode odor identity, concentration, novelty, and timing, particularly in realistic environments.

Feedback inhibition and its control in an insect olfactory circuit

Inhibitory neurons play critical roles in regulating and shaping olfactory responses in vertebrates and invertebrates. In insects, such roles are performed by relatively few neurons, which can be interrogated efficiently, revealing fundamental principles of olfactory coding. With electrophysiological recordings from the locust and a large-scale biophysical model, we analyzed the properties and functions of the giant GABAergic neuron (GGN), a unique neuron that plays a central role in structuring olfactory codes in the locust brain. Analysis of our in vivo recordings and simulations of our model of the olfactory network suggest that the GGN extends the dynamic range of Kenyon cells (high-order neurons in a brain area analogous to the vertebrate piriform cortex and which fire spikes when the animal is presented with an odor pulse), which leads us to predict the existence of a yet undiscovered olfactory pathway. Our analysis of GGN–intrinsic properties, inputs, and outputs, in vivo and in silico, reveals basic new features of this critical neuron and the olfactory network that surrounds it. Together, results of our in vivo recordings and large-scale realistic computational modeling provide a more complete understanding of how different parts of the olfactory system interact.
Oscillatory integration windows in neurons
Oscillatory synchronization of neurons occurs in many brain regions, including the olfactory systems of vertebrates and invertebrates, and is indispensable for precise olfactory coding. One mechanism by which oscillations have been proposed to influence coding is through the creation of cyclic integration windows, i.e., specific times within the oscillation cycle when synaptic input is most efficiently integrated by a postsynaptic neuron. Cyclic integration windows could allow a neuron to respond preferentially to spikes arriving coincidentally from several presynaptic neurons in a specific part of the cycle. Thus, coincidence detection mediated by integration windows could help read precise temporal codes for odors. Phase-specific effects of synaptic inputs have been described in both brain slices and simulations. However, the existence of cyclic integration windows has not been demonstrated, and their functional requirements are unknown.

With paired local field potential (LFP) and intracellular recordings and controlled stimulus manipulations, we directly tested the idea in the locust olfactory system. We focused on the responses of Kenyon cells. We found that inputs arriving in Kenyon cells sum most effectively in a preferred window of the oscillation cycle. With a computational model, we established that the non-uniform structure of noisy activity in the membrane potential helps mediate the process. Further experiments performed in vivo demonstrated that integration windows can form in the absence of inhibition and in a broad range of oscillation frequencies.

Our results establish that cyclic integration windows can be formed from very few ingredients, i.e., oscillatory input and noise in the membrane potential. Given the ubiquity of membrane noise, the mechanisms we
describe likely apply to a wide variety of neurons that receive oscillatory inputs, with or without inhibition and across a range of frequencies. Our results reveal how a fundamental coincidence-detection mechanism in a neural circuit functions to decode temporally organized spiking.

Spatiotemporal coding of individual chemicals by the gustatory system

Four of the five major sensory systems (vision, olfaction, somatosensation, and audition) are thought to be encoded by spatiotemporal patterns of neural activity. The only exception is gustation. Gustatory coding by the nervous system is thought to be relatively simple, i.e., every chemical ('tastant') is associated with one of a small number of basic tastes, and the presence of a basic taste, rather than the specific tastant, is represented by the brain. In mammals as well as insects, five basic tastes are usually recognized: sweet, salty, sour, bitter, and umami. The neural mechanism for representing basic tastes is unclear. The most widely accepted postulate is that, in both mammals and insects, gustatory information is carried through labelled lines of cells sensitive to a single basic taste, that is, in separate channels from the periphery to sites deep in the brain. An alternative proposal is that the basic tastes are represented by populations of cells, with each cell sensitive to several basic tastes.

Testing these ideas requires determining, point-to-point, how tastes are initially represented within the population of receptor cells and how this representation is transformed as it moves to higher-order neurons. However, it has been highly challenging to deliver precisely timed tastants while recording cellular activity from directly connected cells at successive layers of the gustatory system. Using a new moth preparation, we designed a stimulus and recording system that allowed us to fully characterize the timing of tastant delivery and the dynamics of the tastant-elicited responses of gustatory receptor neurons and their monosynaptically connected second-order gustatory neurons, before, during, and after tastant delivery.

Surprisingly, we found no evidence consistent with a basic taste model of gustation. Instead, we found that the moth's gustatory system represents individual tastant chemicals as spatiotemporal patterns of activity distributed across the population of gustatory receptor neurons. We further found that the representations are transformed substantially, given that many types of gustatory receptor neurons converge broadly upon follower neurons. The results of our physiological and behavioral experiments suggest that the gustatory system encodes information not about basic taste categories but rather about the identities of individual tastants. Furthermore, the information is carried not by labelled lines but rather by distributed, spatiotemporal activity, which is a fast and accurate code. The results provide a dramatically new view of taste processing.

Publications

Collaborators

• Tamás Balla, MD, PhD, Section on Molecular Signal Transduction, NICHD, Bethesda, MD
• Maxim Bazhenov, PhD, Howard Hughes Medical Institute, The Salk Institute for Biological Studies, La Jolla, CA

Contact

For more information, email stopferm@mail.nih.gov or visit https://www.nichd.nih.gov/research/atNICHD/Investigators/stopfer or https://dir.ninds.nih.gov/Faculty/Profile/mark-stopfer.html.
The group currently has two main interests: (1) identification and characterization of small noncoding RNAs; and (2) identification and characterization of small proteins of less than 50 amino acids. Both small RNAs and small proteins have been overlooked because they are not detected in biochemical assays, and the corresponding genes are missed by genome annotation and are poor targets for genetic approaches. However, both classes of small molecules are now being found to have important regulatory roles in organisms ranging from bacteria to humans.

Identification and characterization of small regulatory RNAs
During the past 20 years, we have carried out several different systematic screens for small regulatory RNAs (sRNAs) in *Escherichia coli*, which showed that sRNAs are encoded by diverse loci including the 5’ and 3’ UTRs of mRNAs [Reference 1]. The screens included computational searches for conservation of intergenic regions and direct detection after size selection or co-immunoprecipitation with the RNA-binding protein Hfq. We are currently examining sRNA expression using several deep-sequencing approaches to further extend our identification of sRNAs in a range of bacteria species.

A major focus for the group has been to elucidate the functions of the sRNAs that we and others identified. Early on, we showed that the OxyS RNA, whose expression is induced in response to oxidative stress, acts to repress translation through limited base pairing with target mRNAs. We discovered that OxyS action depends on the Sm-like Hfq protein, which acts as a chaperone to facilitate OxyS RNA base pairing with its target mRNAs. We also started to explore the role of ProQ, a second RNA chaperone in *E. coli*. As shown in Figure 1, by comparing the sRNA–mRNA interactomes by deep sequencing, we found that ProQ and Hfq have overlapping as well as competing roles in the cell [Reference 2].

It is clear that Hfq-binding sRNAs, which act through limited base pairing, are integral to many different stress responses in *E. coli* and other bacteria as well as during the interaction between bacteria and bacteriophage [Reference 3]. For example, we showed that the
Spot 42 RNA, whose levels are highest when glucose is present, plays a broad role in catabolite repression by directly repressing genes involved in central and secondary metabolism, redox balancing, and the consumption of diverse non-preferred carbon sources. Similarly, we discovered that a Sigma(E)–dependent sRNA, MicL, transcribed from a promoter located within the coding sequence of the cutC gene, represses synthesis of the lipoprotein Lpp, the most abundant protein in the cell, to oppose membrane stress. We found that the copper-sensitivity phenotype previously ascribed to inactivation of the cutC gene is actually derived from the loss of MicL and from elevated Lpp levels. This observation raises the possibility that other phenotypes currently attributed to protein defects are the result of deficiencies in unappreciated regulatory RNAs. Studies to determine the factors that direct the cleavage of MicL, and likely other sRNAs, from the 3′ untranslated regions (UTRs) of mRNAs, showed that 3′ stem-loops are critical for the very specific processing [Reference 4]. Most recently while characterizing the response to limited magnesium, we found that the adjacently encoded MgrR sRNA and MgtS small protein both down regulate the pitA–encoded cation-phosphate symporter to increase intracellular magnesium levels [Reference 5].

In addition to sRNAs that act via limited base pairing, we have been interested in regulatory RNAs that act by other mechanisms. For example, early work showed that the 6S RNA binds to and modulates RNA polymerase by mimicking the structure of an open promoter. In another study, we discovered that a broadly conserved RNA structure motif yybP-ykoY motif, found in the 5′-UTR of the mntP gene encoding a manganese exporter, directly binds manganese, resulting in a conformation that liberates the ribosome-binding site.

FIGURE 1. RNA–RNA interactomes of ProQ and Hfq reveal overlapping and competing roles.

A comparison between the RNA–RNA interactomes of the well-characterized Hfq and the understudied ProQ RNA chaperones revealed that a significant fraction of the RNA–RNA pairs on ProQ are also found on Hfq. Interestingly, sRNA–mediated regulation and the population of RNAs bound by Hfq and ProQ are affected by growth conditions.
Further studies to characterize other Hfq- and ProQ-binding RNAs and their physiological roles and evolution, as well as regulatory RNAs that act in ways other than base pairing, are ongoing.

**Identification and characterization of small proteins**

In our genome-wide screens for sRNAs, we found that several short transcripts do indeed encode small proteins. The correct annotation of the genes encoding the smallest proteins is one of the biggest challenges of genome annotation. However, even though small-protein genes have been largely missed, the few small proteins studied in bacterial and mammalian cells have been shown to have important functions in regulation, signaling, and cellular defenses [Reference 6]. We thus established a project to identify and characterize proteins of less than 50 amino acids.

We first used sequence conservation and ribosome binding-site models to predict genes encoding small proteins of 16–50 amino acids in the intergenic regions of the *E. coli* genome. We tested expression of these predicted as well as previously annotated small proteins by integrating the sequential peptide affinity tag directly upstream of the stop codon on the chromosome and assaying for synthesis using immunoblot assays. The approach confirmed that 20 previously annotated and 18 newly discovered proteins of 16–50 amino acids are synthesized. We recently carried out a complementary approach based on genome-wide ribosome profiling of ribosomes arrested in start codons to identify many additional candidates; we confirmed the synthesis of 38 of these small proteins by chromosomal tagging. These studies, together with the work of others, documented that *E. coli* synthesizes over 150 small proteins.

Many of the initially discovered proteins were predicted to consist of a single transmembrane alpha-helix and, in biochemical fractionation, were found to be in the inner membrane. Assays of topology-reporter fusions and strains with defects in membrane insertion proteins revealed that, despite their diminutive size, small membrane proteins display considerable diversity in topology and insertion pathways. Additionally, systematic assays for the accumulation of tagged versions of the proteins showed that many small proteins accumulate under specific growth conditions or after exposure to stress. We also generated and screened bar-coded null mutants and identified small proteins required for resistance to cell envelope stress and acid shock.

We now are using the tagged derivatives and information about synthesis and subcellular localization, and, to elucidate the functions of the small proteins, are employing many of the approaches the group used to characterize the functions of sRNAs. The combined approaches are beginning to yield insights into how the small proteins act in *E. coli*. We found that synthesis of a 42-amino acid protein, now denoted MntS, is repressed by high levels of manganese. Correspondingly, the lack of MntS leads to reduced activities of manganese-dependent enzymes under manganese-poor conditions, while overproduction of MntS leads to very high intracellular manganese and bacteriostasis under manganese-rich conditions. These and other phenotypes led us to propose that MntS modulates intracellular manganese levels, possibly by inhibiting the manganese exporter MntP. We also showed that the 31-amino acid inner-membrane protein MgtS (formerly denoted YneM), whose synthesis is induced by very low magnesium, acts to increase intracellular magnesium levels and maintain cell integrity upon magnesium depletion. Upon development of a functional tagged derivative of MgtS, we found that MgtS interacts with MgtA to increase the levels of this P-type ATPase magnesium transporter under magnesium-limiting conditions. Correspondingly, the effects of MgtS upon magnesium limitation are lost in an mgtA mutant, and MgtA overexpression can suppress the mgtS phenotype. MgtS stabilization of MgtA provides an additional layer of regulation of this tightly controlled...
magnesium transporter. Most recently we found that MgtS also interacts with and modulates the activity of a second protein, the PitA cation-phosphate symporter, to further increase intracellular magnesium levels [Reference 5].

We also discovered the 49–amino acid inner-membrane protein AcrZ (formerly named YbhT), whose synthesis is increased in response to noxious compounds such as antibiotics and oxidizing agents, associates with the AcrAB–ToIC multidrug efflux pump, which confers resistance to a wide variety of antibiotics and other compounds. Co-purification of AcrZ with AcrB, in the absence of both AcrA and ToIC, two-hybrid assays, and suppressor mutations indicate that the interaction occurs through the inner-membrane protein AcrB. Mutants lacking AcrZ are sensitive to many, but not all, the antibiotics transported by AcrAB–ToIC. Such differential antibiotic sensitivity suggests that AcrZ enhances the ability of the AcrAB–ToIC pump to export certain classes of substrates. Detailed structural and mutational studies are now giving insight into how AcrZ affects AcrB, and recently showed that AcrZ and cardiolipin cooperate to allosterically modulate AcrB activity.

This work, together with our ongoing studies of other small proteins and related findings by others in eukaryotic cells, supports our hypothesis that small proteins are an overlooked class of important regulators.

**Publications**


**Collaborators**

- Katarzyna J. Bandyra, PhD, *University of Cambridge*, Cambridge, United Kingdom
- Allen R. Buskirk, PhD, *Johns Hopkins Medical Institute*, Baltimore, MD
- Dijun Du, PhD, *School of Life Science and Technology*, ShanghaiTech University, Pudong, China
- Susan Gottesman, PhD, *Laboratory of Molecular Biology, Center for Cancer Research*, NCI, Bethesda, MD
- Matthew R. Hemm, PhD, *Department of Biological Sciences*, Towson University, Towson, MD
- Syma Khalid, PhD, *School of Chemistry*, University of Southampton, Southampton, United Kingdom
- Ben F Luisi, PhD, *Department of Biochemistry*, University of Cambridge, Cambridge, United Kingdom
- Shu-Bing Qian, PhD, *Cornell University*, Ithaca, NY
- Shabalina A. Shabalina, PhD, *National Center for Biotechnology Information, National Library of Medicine*, Bethesda, MD
• Jörg Vogel, Dr rer nat, *Institute of Molecular Infection Biology, Universität Würzburg, Würzburg, Germany*
• Henry Zhang, PhD, *Bioinformatics and Scientific Programming Core, NICHD, Bethesda, MD*

**Contact**
For more information, email *storz@helix.nih.gov* or visit *http://storz.nichd.nih.gov*. 
Molecular Genetics of Endocrine Tumors and Related Disorders

In the project that was started in the late 1990s, our approach has been to study patients with rare endocrine conditions, mostly inherited, identify the causative genes, and then study the signaling pathways involved in the hope of translating the derived knowledge into new therapies for such patients. The derived knowledge could also be generalized to conditions that are not necessarily inherited, e.g., to more common tumors and diseases caused by defects in these molecular pathways. The approach has indeed led to fruitful research over the last quarter of century. Our first studies led to the identification of the main regulator of the cAMP signaling pathway, the regulatory subunit type 1A (R1a) of protein kinase A (PKA, encoded by the PRKAR1A gene on chromosome 17q22-24), as responsible for primary pigmented nodular adrenocortical disease (PPNAD) and the Carney complex, a multiple endocrine neoplasia (MEN), whose main endocrine manifestation is PPNAD. We then focused on clinically delineating the various types of primary bilateral adrenal hyperplasias (BAHs). We described isolated micronodular adrenocortical disease (iMAD), a disorder likely to be inherited in an autosomal dominant manner and unrelated to the Carney complex or to other MENs. The identification of PRKAR1A mutations in PPNAD led to the recognition that non-pigmented forms of BAH exist, and a new nomenclature was proposed, which we first suggested in 2008 and is since used worldwide.

In 2006, a genome-wide association (GWA) study led to the identification of mutations in the phosphodiesterases (PDE) PDE11A, a dual specificity PDE, and in PDE8B, a cAMP–specific PDE (encoded by the PDE11A and PDE8B genes, respectively) in iMAD. Following the establishment of cAMP/PKA involvement in PPNAD and iMAD, we and others discovered that elevated cAMP levels and/or PKA activity and abnormal PDE activity may be found in most benign adrenal tumors (ADTs), including the common adrenocortical adenoma (ADA). We then found PDE11A and PDE8B mutations or functional variants thereof in adrenocortical cancer (ACA) and in other forms of adrenal hyperplasia such as massive macronodular adrenocortical disease (MMAD), also known as ACTH–independent adrenocortical hyperplasia (MMAD/AIMAH). Germline PDE11A sequence variants may also predispose to testicular cancer (testicular germ-cell tumors or TGCTs) and prostate cancer, indicating a wider role of this tumor-formation pathway in
cAMP–responsive, steroidogenic, or related tissues. Ongoing work with collaborating NCI laboratories aims to clarify the role of PDE in the predisposition to these tumors. However, it is clear from these data that there is significant pleiotropy of PDE11A and PDE8B defects. The histomorphological studies that we performed on human adrenocortical tissues from patients with these mutations showed that iMAD is highly heterogeneous and thus likely to be caused by defects in various genes of the cAMP/PKA signaling pathway or its regulators and/or downstream effectors.

Similarly, the G protein–coupled receptor (GPCR)–linked MMAD/AIMAH disease includes a range of adrenal phenotypes, from those very similar to iMAD to primary bimorphic adrenocortical disease (PBAD) and McCune-Albright syndrome, which is caused by somatic mutations in the GNAS gene (encoding the G protein–stimulatory subunit alpha [Gsa]). Although a few of the patients with MMAD/AIMAH have germ line PDE11A, PDE8B, or somatic GNAS mutations, others have mutations in the genes encoding germline fumarate hydratase (FH), menin (MEN1), or adenomatous polyposis coli (APC), pointing to the range of possible pathways that may be involved. Particularly interesting among these are FH mutations that are associated with mitochondrial oxidation defects linked to adrenomedullary tumors, which led us to investigate a disorder known as the Carney Triad. The Carney Triad is the only known disease that, among its clinical manifestations, has both adrenocortical (ADA, MMAD/AIMAH) and medullary tumors (pheochromocytomas [PHEOs] and paragangliomas [PGLs]), in addition to hamartomatous lesions in various organs (pulmonary chondromas and pigmented and other skin lesions) and a predisposition to gastrointestinal stromal tumors or sarcomas (GISTs). A subgroup of patients with PHEOs, PGLs, and GISTs were found to harbor mutations in succinate dehydrogenase (SDH) subunits B, C, and D (encoded by the SDHB, SDHC, and SDHD genes, respectively); the patients also rarely have adrenocortical lesions, ADAs, and/or hyperplasia, and their disease is known as the dyad or syndrome of PGLs and GISTs and is now widely known as the Carney-Stratakis syndrome (CSS).

In 2013, MMAD/AIMAH was renamed primary macronodular adrenocortical hyperplasia (PMAH) after it was discovered that it depends on adrenoglandular ACTH production, at least occasionally. As part of this work, a new gene (ARMC5) was identified that, when mutated, causes more than a third of the known PMAH cases. The function of the gene is unknown, and we thus embarked on a project to characterize it further, including studying mouse, fruit fly, and fish models. The ARMC5 gene has a beta-catenin–like motif.

Although PPNAD appears to be less heterogeneous and is mostly caused by PRKAR1A mutations, up to one third of patients with the classic features of PPNAD do not have PRKAR1A mutations, deletions, or 17q22–24 copy-number variant (CNV) abnormalities. A subset of these patients may have defects in other molecules of the PKA holoenzyme, and studying them is important for understanding how PKA works as well as the
tissue specificity of each defect. For patients with disorders that are yet to be elucidated on a molecular level, we continue to delineate the phenotypes and identify the responsible genetic defects through a combination of genomic and transcriptomic analyses. Recently, we identified genes encoding two other subunits of PKA as involved in endocrine tumors: \textit{PRKACA} in BAH and \textit{PRKACB} in a form of the Carney complex that is not associated with \textit{PRKAR1A} mutations. Our laboratory is now investigating the two genes.

Animal model studies are essential for the investigation and confirmation of each of the identified new genes in disease pathogenesis. Furthermore, such studies provide insight into function that can be tested quickly in human samples for confirmation of its relevance to human disease. One excellent example of such a bench-to-bedside (and back) process was our recent identification, from a variety of animal experiments, of Wingless/int (Wnt) signaling as one of the pathways interacting with cAMP/PKA in the adrenal cortex. We continue to investigate the pathways involved in early events in tumor formation in the adrenal cortex and/or the tissues affected by germline or somatic defects of the cAMP/PKA and related endocrine signaling defects, employing animal models and transcriptomic and systems-biology analyses. Understanding the role of the other PKA subunits in this process is essential. An example of the combined use of whole genomic tools, transcriptomic analysis, and mouse and zebrafish models to investigate the function of a gene or a pathway is the ongoing work on the Carney Triad.

We continue to accrue patients under several clinical protocols, identify unique patients and families with rare phenotypes, and/or explore (mostly on a collaborative basis) various aspects of endocrine and related diseases. Paramount to these investigations is the availability of modern genetic tools such as copy-number variation (CNV) analysis, comparative genomic hybridization (CGH), whole-exome sequencing (WES), and DNA sequencing (D-seq). As part of the clinical protocols, much clinical research consists mostly of observations of new associations, description of novel applications or modifications, and improvements in older diagnostic methods, tests, or imaging tools, a particularly fruitful area of research, especially for our clinical fellows, who matriculate at our laboratory during their two-year research time. The approach also leads to important new discoveries, which may steer us into new directions.

One such discovery was our recent identification of the defect that explains the vast majority of cases of early pediatric overgrowth or gigantism. What regulates growth, puberty, and appetite in children and adults is poorly understood. We identified the gene \textit{GPR101}, encoding a G protein–coupled receptor, that was overexpressed in patients with elevated growth hormone (GH). Patients with \textit{GPR101} defects have a condition that we called \textit{X-LAG}, for X-linked acrogigantism, is caused by Xq26.3 genomic duplication, and is characterized by early-onset gigantism resulting from excess GPR101 function and consequent elevation of GH. Another recent discovery was the identification of \textit{SGPL1} (sphingosine-1-phosphate lyase 1) deficiency in patients with primary adrenocortical insufficiency.

The last years (2018–2020) followed the successes of the previous years: The Section members were again very productive, publishing at leading journals, and contributing new and important knowledge on the clinical and molecular genetics of the conditions and syndromes that were described in the previous four years. But a transition is evident: as the era of rare diseases gene discovery comes to an end, with more than half of them now molecularly elucidated, and in excess of 30 genetic associations studied by the Section's researchers over the last 25 years (https://irp.nih.gov/pi/constantine-stratakis), the Section follows the shift that was first proposed in 2016: the clinical protocols that focused on Carney complex (1995–2020), adrenal hyperplasia (2000–2020),
and other adrenal disorders were closed in 2020, as their main goals were accomplished (i.e., elucidation of the main genetic causes leading to these tumors). We replaced these studies with disease-specific, new molecular treatment trials (e.g., Pegvisomant® treatment of children with gigantism, supported by Pfizer, Inc) and research on common disorders that relate to the rare diseases and their molecular pathways that we have been investigating (e.g., clinical and molecular characteristics of primary aldosteronism in Blacks, a collaborative program with the University of Michigan, Ann Arbor and others).

In the laboratory, too, our emphasis is on the identification of molecular targets that may lead to clinical trials that we will implement at the NIH CRC. We have already identified compounds in both our PRKACA and GPR101 screening efforts. We are now proceeding with characterizing the compounds in the laboratory and, soon, in animal models. The work would not have been done without access to NIH’s Center for Advancing Translational Sciences (NCATS) and its Chemical Genomics Center (now Early Translation Branch or ETB [www.ncats.nih.gov/etb]), as well as several international collaborators.

Carney complex (CNC) genetics
We collected families with CNC and related syndromes from several collaborating institutions worldwide. Through genetic linkage analysis, we identified loci harboring genes for CNC on chromosomes 2 (2p16) and 17 (17q22–24). The PRKAR1A gene on 17q22–24, the gene responsible for CNC in most cases of the disease, appears to undergo loss of heterozygosity in at least some CNC tumors. PRKAR1A is also the main regulatory subunit (subunit type 1-alpha) of PKA, a central signaling pathway for many cellular functions and hormonal responses. We increased the number of CNC patients in genotype-phenotype correlation studies, which are expected to provide insight into the complex biochemical and molecular pathways regulated by PRKAR1A and PKA. We expect to identify new genes by ongoing genome-wide searches for patients and families who do not carry PRKAR1A mutations.

Mutations in PRKAR1A and protein kinase A activity in other diseases
We are investigating the functional and genetic consequences of PRKAR1A mutations in cell lines from a variety of tumors. We measure both cAMP and PKA activity in the cell lines, along with the expression of the other subunits of the PKA tetramer. We work to identify PRKAR1A–interacting mitogenic and other growth-signaling pathways in cell lines expressing PRKAR1A constructs and/or mutations. Several genes that regulate PKA function and increase cAMP–dependent proliferation and related signals may be altered in the process of endocrine tumorigenesis initiated by a mutant PRKAR1A, a gene with important functions in the cell cycle and in chromosomal stability.

In 2018, we were successful in obtaining funded through a Uniformed Services University of the Health Sciences Award on the “Genetics of human susceptibility to infections and/or complications of Zika virus: variants of the cyclic AMP-dependent PKA pathway.” The resulting publication described an association between Zika virus disease burden and certain variants of genes involved in the cAMP signaling pathway.

Prkar1a+/− and related animal models
Several years ago, we developed a Prkar1a knockout mouse floxed by a lox-P system for the purpose of generating, first, a novel Prkar1a+/− and, second, knockouts of the Prkar1a gene in a tissue-specific manner after crossing the new mouse model with mice expressing the cre protein in the adrenal cortex, anterior lobe of the
FIGURE 1.

A. After a high-fat diet (HFD), Prkar2a knockout (KO) mice were leaner than wild-type (WT).

B, C & D. Female and male KO mice remained leaner as they aged and did not develop a fatty liver after a HFD.

E & F. Prkar2a expression was mapped to the medial habenula (MHb) and, in part, the leaner phenotype was the result of reduced HFD intake.

G. Female (shown) and male KO mice (not shown) run more than twice as much as WT during home cage running wheel access.

H & I. Direct injection of Prkar2a into the MHb reduced voluntary running* to the levels of WT and restored sucrose preference. *Voluntary running activity was graphed in bins of 30 minutes over a two-week period.

pituitary, and thyroid gland. The heterozygote mouse develops several tumors reminiscent of the equivalent human disease. We have now developed new crosses that demonstrate protein kinase A subunit involvement in additional phenotypes. An example of the ongoing work using PKA–subunit animal models is the work on the Prkar2a mouse model (Figure 1). Ongoing work with several animal crosses is investigating various aspects of PKA subunit functions and the possible involvement of cAMP–pathway perturbations in several pathophysiologic and/or disease-related states.

The Prkar2a−/− mouse is involved in motivation to exercise and has preference for certain foods.

The habenula (Hb) is a bilateral, evolutionarily conserved epithalamic structure connecting forebrain and midbrain structures that has gained attention for its roles in depression, addiction, rewards processing, and motivation. Of its two major subdivisions, the medial Hb (MHb) and lateral Hb (LHb), MHb circuitry and function are poorly understood compared with those of the LHb. Prkar2a encodes the cAMP–dependent protein kinase (PKA) regulatory subunit IIα (RIIα), a component of the PKA holoenzyme, at the center of one of the major cell-signaling pathways conserved across systems and species. Type 2 regulatory subunits (RIIα, RIIβ) determine
the subcellular localization of PKA, and, unlike other PKA subunits, Prkar2a shows minimal brain expression except in the Mhb. We previously showed that RIIα-knockout (RIIα-KO) mice resist diet-induced obesity. We more recently reported that RIIα-KO mice consume less palatable, “rewarding” foods and are more motivated for voluntary exercise. Prkar2a deficiency led to decreased habenular PKA enzymatic activity and impaired dendritic localization of PKA catalytic subunits in MHB neurons. Re-expression of Prkar2a in the Hb rescued this phenotype, confirming differential roles for Prkar2a in regulating the drives for palatable foods and voluntary exercise. Our findings show that, in the MHB, decreased PKA signaling and dendritic PKA activity reduce motivation for palatable foods, while enhancing the motivation for exercise, a desirable combination of behaviors (Figure 1).

Genes encoding phosphodiesterase (PDE) in endocrine and other tumors
In patients who did not exhibit CNC or have PRKAR1A mutations but presented with bilateral adrenal tumors similar to those in CNC, we found inactivating mutations of the PDE11A gene, which encodes phosphodiesterase-11A (PDE11A), an enzyme that regulates PKA in the normal physiologic state. Phosphodiesterase 11A is a member of a 22 gene–encoded family of proteins that break down cyclic nucleotides that control PKA. PDE11A appears to act as a tumor suppressor such that tumors develop when its action is abolished. In what proved to be the first cases in which mutated PDE was observed in a genetic disorder predisposing to tumors, we found pediatric and adult patients with bilateral adrenal tumors. Recent data indicate that PDE11A sequence polymorphisms may be present in the general population. The finding that genetic alterations of such a major biochemical pathway may be associated with tumors in humans raises the reasonable hope that drugs that modify PKA and/or PDE activity may eventually be developed to treat both CNC patients and those with other, non-genetic, adrenal tumors, and perhaps other endocrine tumors. After the identification of a patient with a PDE8B mutation and Cushing's syndrome, additional evidence emerged that yet another cAMP–specific PDE is involved in endocrine conditions. We also studied both Pde11a and Pde8b animal models.

Genetic investigations into other adrenocortical diseases and related tumors
Through collaborations, we: (1) apply general and pathway-specific microarrays to a variety of adrenocortical tumors, including single adenomas and MMAD, to identify genes with important functions in adrenal oncogenetics; (2) examine candidate genes for their roles in adrenocortical tumors and development; and (3) identify additional genes that play a role in inherited pituitary, adrenocortical, and related diseases.

This past year, in collaboration with a group in France, we investigated the genetic defects in GIP–dependent Cushing's syndrome, which is caused by ectopic expression of glucose-dependent insulinotropic polypeptide receptor (GIPR) in cortisol-producing adrenal adenomas or in bilateral macronodular adrenal hyperplasias. We performed molecular analyses on the adrenocortical adenomas and bilateral macronodular adrenal hyperplasias obtained from 14 patients with GIP–dependent adrenal Cushing's syndrome and one patient with GIP–dependent aldosteronism. GIPR expression in all adenoma and hyperplasia samples occurred through transcriptional activation of a single allele of the GIPR gene. While no abnormality was detected in proximal GIPR promoter methylation, we identified somatic duplications in chromosome region 19q13.32, which contains the GIPR locus, in the adrenocortical lesions derived from three patients. In two adenoma samples, the duplicated 19q13.32 region was rearranged with other chromosome regions, whereas a single tissue sample with hyperplasia had a 19q duplication only. Our French collaborators showed that juxtaposition with cis-acting regulatory sequences, such as glucocorticoid-response elements, in the newly identified genomic environment drives abnormal expression of the translocated GIPR allele in adenoma cells.
We continue to work on identifying new genetic defects in other forms of adrenal tumors and/or hyperplasias. We have now identified variants in PRKAR1B and PRKACB predisposing to adrenal tumors, in addition to PRKACA, ARMCS, and of course PRKAR1A.

**Genetic investigations into pituitary tumors, X-LAG, other endocrine neoplasias, and related syndromes**

In collaboration with several other investigators at the NIH and elsewhere, we are investigating the genetics of CNC– and adrenal-related endocrine tumors, including childhood pituitary tumors, related or unrelated to PRKAR1A mutations. As part of this work, we identified novel genetic abnormalities.

We identified the gene **GPR101**, which encodes an orphan G protein–coupled receptor (GPCR) and is overexpressed in patients with elevated growth hormone (GH) or gigantism. Patients with **GPR101** defects have a condition that we called X-LAG, for X-linked acrogigantism, is caused by Xq26.3 genomic duplication, and is characterized by early-onset gigantism resulting from excessive GPR101 function and consequent GH excess. To find additional patients with this disorder, we collaborated with a group in Belgium, but all the molecular work for gene identification was carried out here at the NIH. We found that the gene is expressed in areas of the brain that regulate growth, and we are actively investigating small-molecule compounds that may bind to GPR101 (unpublished).

In addition, we studied patients with pediatric Cushing disease (CD), which results from corticotropin (ACTH)–secreting pituitary tumors, as part of our studies on Cushing’s syndrome. Almost everything known today in the literature about pediatric CD, from its molecular investigations to its diagnosis and treatment, is derived from work that was done at the NIH. This laboratory is currently intensely involved in the identification of genetic defects that predispose to pediatric CD. Last year, we reported **CABLES1** (encoding a cyclin-dependent kinase-binding protein) and **USP8** (encoding ubiquitin carboxyl-terminal hydrolase 8) mutations in patients with CD (**CABLES1**) and/or their tumors (**USP8**).
Genetic investigations into the Carney Triad, other endocrine neoplasias, and related syndromes and into hereditary paragangliomas and related conditions

As part of a collaboration with other investigators at the NIH and elsewhere (including an international consortium organized by our laboratory), we are studying the genetics of the Carney Triad, a rare syndrome that predisposes to adrenal and other tumors, and of related conditions (associated with gastrointestinal stromal tumors [GIST]). In the course of our work, we identified a patient with a new syndrome, known as the paraganglioma and gastrointestinal stromal tumor syndrome (or Carney-Stratakis syndrome), for which we found mutations in the genes encoding succinate dehydrogenase (SDH) subunits A, B, C, and D. In another patient, we found a novel germline mutation in the tyrosine kinase–encoding PDFGRA gene. In collaboration with a group in Germany, we identified an epigenetic defect (methylation of the SDHC gene) that may be used diagnostically to identify patients with the Carney Triad.

Clinical investigations into the diagnosis and treatment of adrenal and pituitary tumors

Patients with adrenal tumors and other types of Cushing's syndrome (and occasionally other pituitary tumors) come to the NIH Clinical Center for diagnosis and treatment. Ongoing investigations focus on: (1) the prevalence of ectopic hormone receptor expression in adrenal adenomas and PMAH/MMAD; (2) the diagnostic use of high-sensitivity magnetic resonance imaging for earlier detection of pituitary tumors; and (3) the diagnosis, management, and postoperative care of children with Cushing's syndrome and other pituitary tumors.

Clinical and molecular investigations into other pediatric genetic syndromes

Mostly in collaboration with several other investigators at the NIH and elsewhere, we are conducting work on pediatric genetic syndromes seen in our clinics and wards. One such example is the recent identification of SGPL1 defects in patients with primary adrenal insufficiency.

Additional Funding

- INSERM, Paris, France (Co-Principal Investigator): “Clinical and molecular genetics of Carney complex,” 06/2003–present
- Several small grants supporting staff members from France, Brazil, Greece, Spain, and elsewhere
- Bench-to-Bedside 2017 Award: “Therapeutic targets in African Americans with primary aldosteronism”
- Gifts on Cushing’s syndrome research (various private donations)
- Pfizer #W1215907 2017–2018 US ASPIRE ENDOCRINE study titled “Characterization of GPR101-mediated growth regulation and receptor deorphanization”
- Uniformed Services University of the Health Sciences 2018 Award “Genetics of human susceptibility to infections and/or complications of Zika virus: variants of the cyclic AMP-dependent PKA pathway”

Publications

2. Drougat L, Settas N, Ronchi CL, Bathon K, Calebiro D, Maria AG, Haydar S, Voutetakis A, London E,


**Collaborators**

- Albert Beckers, MD, *Université de Liège, Liège, Belgium*
- Jerome Bertherat, MD, PhD, *Service des Maladies Endocriniennes et Métaboliques, Hôpital Cochin, Paris, France*
- Sosipatros Boikos, MD, *Medical College of Virginia, Medical Oncology, Richmond, VA*
- Stephan Bornstein, MD, PhD, *Universität Dresden, Dresden, Germany*
- Isabelle Bourdeau, MD, *Université de Montréal, Montréal, Canada*
- J. Aidan Carney, MD, PhD, *Mayo Clinic, Rochester, MN*
- Nickolas Courkoutsakis, MD, PhD, *University of Thrace, Alexandroupolis, Greece*
- Jacques Drouin, PhD, *Université de Montréal, Montréal, Canada*
- Jennifer Gourgari, MD, *Georgetown University, Washington, DC*
- Margaret F. Keil, PhD, RN, CRNP, *Office of the Clinical Director, NICHD, Bethesda, MD*
- Lawrence Kirschner, MD, PhD, *James Cancer Hospital, Ohio State University, Columbus, OH*
- Andre Lacroix, MD, PhD, *Centre Hospitalier de l'Université de Montréal, Montréal, Canada*
- Giorgios Papadakis, MD, MPH, *University of Crete, Heraklion, Greece*
- Nickolas Patronas, MD, *Diagnostic Radiology, Clinical Center, NIH, Bethesda, MD*
- Erwin Van Meir, PhD, *Emory University, Atlanta, GA*
- Antonios Voutetakis, MD, PhD, *University of Athens, Athens, Greece*

**Contact**

For more information, email *stratak@mail.nih.gov* or visit [http://segen.nih.gov](http://segen.nih.gov).
Biomedical Optics Technologies for Vascular and Metabolic Disease

Through the advancement of models, methods, and devices that utilize the interaction of light with biological tissue, we strive to develop non-invasive techniques that can help guide therapy and aid in clinical decision making. The techniques are used to perform real-time quantitative measurements of clinically relevant information, including tissue blood flow, oxygen extraction, and body/tissue composition. Our research seeks to move these technologies from “bench to bedside,” where they can be applied to clinical problems, including vascular and metabolic disease.

Non-invasive optical imaging technology for characterization of tissue hemodynamics and composition

Aberrations in tissue hemodynamics (i.e., blood flow, oxygenation, and oxygen metabolism) and tissue composition are observed in a wide variety of diseases, including cancer, cardiovascular disease, diabetes, and neurodegenerative disorders. Malignant tumors, for example, lead to the higher blood flow and oxygen consumption needed to fuel rapid cellular growth. In peripheral arterial disease (PAD), long-term buildup of plaque along the artery walls causes reduced perfusion in peripheral circulation. In all such conditions, techniques that can characterize tissue hemodynamics and composition can improve strategies for early diagnosis, screening, and treatment-response monitoring.

There are several clinically accepted ways to assess tissue hemodynamics and composition. Techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) have been used for measuring blood flow through tissue. Additionally, MRI and dual X-ray absorptiometry (DXA) can be used to determine body composition and distinguish between lean and soft tissue. However, these techniques are time- and resource-intensive and can only be accessed in a hospital setting. As a result, patients typically undergo such assessments only after the onset of severe symptoms. As alterations in vascular health occur gradually over time, there is a need for technologies that can assess tissue hemodynamics and composition at the point of care.

To characterize tissue hemodynamics or composition in portable form factors, near-infrared spectroscopy (NIRS) techniques have emerged.
as lower-cost and non-invasive alternatives. Techniques such as diffuse optical spectroscopic imaging (DOSI) or spatial frequency domain imaging (SFDI) can quantify the concentration of hemoglobin, water, and bulk lipids. In addition to the compositional information that can be obtained, continuous measurements with these techniques can also assess tissue hemodynamics in terms of the delivery and consumption of oxygen by observing changes in oxy- and deoxy- hemoglobin concentration. While the techniques employ similar principles to measure similar information, they differ in terms of the field of view and depth of tissue interrogated. Another subset of NIRS techniques can quantify blood flow, which is necessary to more accurately characterize tissue metabolic activity. Technologies such as laser speckle imaging (LSI), laser Doppler flowmetry (LDF), and diffuse correlation spectroscopy (DCS) all measure fluctuations in intensity caused by light scattering events from moving particles such as red blood cells, in order to provide quantitative measure of blood flow.

Optical techniques have proven safe and effective in assessing blood flow and tissue oxygenation, but very little is known about the normal range of hemodynamic properties and the degree to which they change as a result of normal physiological variation. It is unclear how much the variations affect standard measurements and whether they contain any novel information. Additionally, tissue composition naturally varies between patients owing to differences in diet and fitness level. Different tissue types have different metabolic activity based on their composition, which will influence overall tissue hemodynamics. The interplay of structure and function necessitates the development and characterization of systems that can characterize both.

Development of a hand-held diffuse optical spectroscopy system

There is an urgent need for more powerful and cost-effective wearable and bedside technologies to meet the needs of healthcare providers and patients for personalized medicine and personal health. However, many conventional strategies are insufficient, and there are clear opportunities for new technologies to meet this rapidly growing demand. Tissue optical spectroscopy is widely used in biomedical research and clinical medicine to characterize perfusion, metabolism, and molecular composition and can potentially provide new approaches to meet this challenge. We and others have developed optical methods for detection, diagnosis, and therapeutic guidance in virtually all major areas of medicine such as cancer, cardiovascular disease, diabetes, metabolic disease, and trauma/critical care. In addition, the diffuse photoplethysmographic (PPG) waveform is essential for assessing heart rate in personal health monitors (e.g. Fitbit, Apple Watch, etc.) as well as in medical devices for tissue oximetry and continuous blood pressure monitoring.

While much of this work is conducted using time-independent, continuous-wave (CW) illumination methods that
only assess changes in light intensity, a significant and growing body of work shows that time-resolved methods, which employ high-speed modulated (e.g., more than 50 MHz) or picosecond-pulsed sources to measure changes in the temporal dispersion of light, have powerful advantages. Unlike CW techniques, which typically do not account for alterations in optical scattering that can occur during the measurement period and between individuals, time-resolved measurements can quantitatively determine optical path length in centimeter-thick, multiply scattering tissues by measuring either the phase velocity or temporal dispersion of modulated or pulsed sources, respectively. As a result, these time-resolved methods, based on modulated light sources, are used to separate light absorption from scattering and provide substantially greater information content than CW intensity alone. Their quantitative features allow for absolute comparisons between individuals and longitudinal monitoring of subjects with exceptional accuracy and precision over many time points. Unfortunately, current time-resolved methods are prohibitively complex, bulky, and expensive, and generally not suitable for compact wearable devices. With advances in complementary metal-oxide semiconductor (CMOS) and vertical cavity surface emitting laser (VCSEL) technologies, it is now possible to overcome these limitations by creating integrated circuits for time-resolved spectroscopy and imaging. Such state-of-the-art, enabling technologies will accelerate the development of new methods and devices with the accuracy, precision, and power of time-resolved techniques, but with the simplicity, low cost, and wearability of CW devices.

The goal of this project is to develop hand-held, easily portable diffuse optical spectroscopy (DOS) systems that perform comparably with previous systems at a fraction of the size and cost. Our primary goal is to develop a DOS system that is driven by a frequency-domain application-specific integrated circuit (fdASIC), which was developed by collaborators at the University of California, Irvine. This will include characterization of the fdASIC performance, design and fabrication of a DOS system driven by the fdASIC, performance characterization and comparison with previously validated DOS systems using known laboratory standards, and in vivo characterization. We will also develop a simpler DOS instrument driven by a commercially available, compact network analyzer in order to streamline the design of the additional optical and electronic components necessary for the fdASIC system.

**Development of a wearable point-of-care monitoring device for pediatric obstructive sleep apnea**

Obstructive sleep apnea (OSA) is the most common type of sleep apnea, in which the blockage of the airway causes breathing to stop involuntarily for ten seconds or more throughout the night during sleep. When
breathing stops, the oxygen level in the blood can drop to harmfully low levels. Pediatric obstructive sleep apnea (POSA) can be especially concerning, with several associated morbidities that can have long-term effects extending into adulthood, including adverse changes in cardiovascular, metabolic, and developmental health. Unfortunately, POSA remains largely underdiagnosed owing to a lack of education about symptoms and limited availability of sleep-medicine physicians. Early diagnosis and treatment are imperative to prevent many of the morbidities.

Polysomnography (PSG), the current standard of care, is expensive, cumbersome, and resource-intensive. At-home sleep apnea tests (HSAT) are less expensive but are equally uncomfortable, prone to user error, and are less effective in children. There is a pressing need for an accurate, robust, unobtrusive alternative for monitoring POSA in the home.

Additionally, the mechanisms of OSA that explain OSA–related outcome measures involving brain health and degeneration are still largely unknown. Approximately 50% of patients with OSA are excessively sleepy during the day, and many develop cardiovascular disease, cerebrovascular disease, and/or cognitive impairment, particularly if untreated. Traditional OSA measures do not predict sleepiness and the apnea-hypopnea index (AHI) and oxygen desaturation index (ODI), measured through standard pulse oximetry, do not typically explain health outcomes, although both hypoxemia and sleep fragmentation are mildly associated.

NIRS is a non-invasive optical technique that can provide direct examination of tissue hemodynamics using near-infrared light in the range of 700 to 1000 nm. Compared with other well-established brain imaging modalities, such as fMRI and PET, the technique has a higher temporal resolution (in order of milliseconds), and provides additional physiological information including heart rate variability and respiration rate. Also, NIRS devices are smaller and can be built into a compact, inexpensive form factor, which is advantageous for ease-of-use and accessibility. NIRS instruments can tolerate subject motion to a greater extent than can fMRI. Such features make the technique ideally suited for studying children, particularly those with problems such as attention-deficit hyperactivity disorder (ADHD), which can make keeping still for long periods of time very challenging. The relationships between NIRS parameters and traditional measurements taken during a sleep study are still
largely unknown. NIRS could provide a better prediction of health outcomes and a greater understanding of brain hypoxia during OSA events.

In conjunction with Amir Gandjbakhche as well as Ashura Buckley, we will incorporate NIRS technologies with standard PSG measurement to understand how optical signals change throughout sleep by comparing physiological signals derived from NIRS with data from PSG and how to develop future optical technologies to provide better patient care that can be used in the home setting. We will test the potential of NIRS technology to diagnose POSA and the response of the brain and tissue during an apnea/hypopnea event and investigate these signals before and after treatment.

Assessment of exercise training and vascular function following COVID-19

The emergence of SARS-CoV-2 as a novel coronavirus at the end of 2019 (COVID-19) resulted in a pandemic that has affected millions of people worldwide. Although clinicians and scientists are gaining an understanding of the various presentations and manifestations of COVID-19, limited reports are available on the recovery trajectory and long-term outcomes faced by survivors. With the increasing number of COVID-19 cases being reported in the United States and worldwide, many patients will require follow-up care related to acute respiratory distress syndrome (ARDS), critical illness, and prolonged hospitalization. Those recovering from ARDS and ICU stays typically experience several negative health outcomes, which include physical and mental deficits and reduced life satisfaction and social health. Effective use of aerobic exercise training (AET) as a cardiorespiratory, rehabilitative intervention could have a high impact on patient functional and quality-of-life outcomes.

Assessment of vascular hemodynamics is critical for evaluating the impact of any exercise treatment. Additionally, there is growing evidence of the negative impact that COVID-19 can have on the vasculature, leading to endothelial dysfunction and the formation of microthrombi. Current methods to assess vascular health are available in both a clinical setting and as commercially available devices for at-home use. Clinical assessments include standard blood panels in addition to diagnostic modalities such as peripheral arterial tonometry (PAT); such approaches are highly informative but can only be performed in a hospital setting, which
limits their accessibility. Consumer-grade devices are significantly more accessible and can even be found in wearable devices such as the Apple Watch or Fitbit. However, the information available to these technologies is limited to standard vital-signs information such as blood pressure, pulse rate, and blood oxygen levels, which is far from a comprehensive assessment of vascular health. Given the chronic nature of the lingering after-effects of COVID-19, the development of technologies that can supply the depth of information comparable to clinical technologies while maintaining the accessibility of consumer-grade devices would be invaluable for characterizing the cardiovascular impact of COVID-19 as well as any prospective treatments.

Optical technologies such as NIRS, which non-invasively assess tissue oxygenation, local oxygen consumption, metabolism, and blood flow, have shown great promise as instruments for determining vascular function. Many of these devices are highly portable and could be attractive options for continuous monitoring of vascular function. In a study led by our collaborator Leighton Chan to investigate the impact of AET as a rehabilitative intervention, we will perform a series of optical measurements to characterize the vascular function of patients who have recovered from a COVID-19 infection and are undergoing an aerobic exercise regimen. In collaboration with Manfred Boehm’s group, we will also evaluate the results acquired by these NIRS methods in conjunction with traditional tests of vascular function, including pulse wave analysis, cardioankle vascular index, and PAT in patients following COVID-19.

Development of a multimodal biosensor for screening and monitoring infectious respiratory diseases

The ongoing COVID-19 pandemic has been a taxing challenge to healthcare systems worldwide. A surge in cases can threaten to overwhelm hospitals’ personnel and resources. The wide range and varying severities of COVID-19 symptoms further add to the challenge of managing the disease. While some cases of COVID-19 are mild and require no further intervention, more severe cases can involve major cardiorespiratory symptoms that could require a ventilator. Rapid, point-of-care tests are valuable for identifying patients who are infected...
with the virus; however, they do not serve as indicators for patient outcomes. A compact method to monitor patient health status and predict patient outcomes could improve our understanding of physiological impact of COVID-19 and alleviate potential strain on hospital resources.

The current standard of care to monitor patient health status revolves around vital-sign parameters such as heart rate, arterial oxygen saturation, respiration rate, and temperature. Some reports have examined personal health devices such as the Apple Watch, Oura Ring, and WHOOP and the continuous health data they all collect, in order to develop models to identify symptomatic patients infected with COVID-19. However, this work still relies on the basic vital sign parameters mentioned above, which only represent a small subset of information on how the body is functioning. Given the growing evidence of the negative impact of COVID-19 on the cardiovascular system, especially the microvasculature, characterization of microvascular health could further improve these predictive models.

NIRS is uniquely positioned to provide distinct information about the delivery and consumption of oxygen in compact form factors. Tissue oxygen saturation, which describes the oxygenation of blood at the tissue and capillary level, is one such metric that can offer valuable insight into microvascular function. In contrast, arterial oxygen saturation, which is normally reported with pulse oximetry, describes the oxygenation of blood coming from the arteries and is therefore an indicator only of oxygen supply. With a sufficiently high sampling rate, the photoplethysmographic (PPG) waveform can also be acquired, which enables NIRS to collect the same vital sign parameters as those collected by commercially available wearables.

We developed a multi-modal optical biosensor capable of real-time, continuous monitoring of tissue oxygenation in addition to vital-sign parameters such as heart rate, respiration rate, and temperature. In order to characterize the performance of our device, we plan to benchmark it to other out-of-the-box wearables. Next, we will measure a cohort of patients with COVID-19 and healthy controls in order to characterize their microvascular health. We believe that including continuous assessment of microvascular health can supplement current patient-monitoring tools, improve understanding of the vascular impact of COVID-19, and aid in the prediction of patient outcomes.
Optical characterization of vascular health in sickle cell disease

Sickle cell disease (SCD) is a disorder that alters the morphology of red blood cells, which negatively impacts oxygen delivery. As a result of the ‘sickling’ of red blood cells, patients diagnosed with SCD can suffer from impaired oxygen delivery and blood flow, which has many negative downstream effects. Over time, the impact of impaired vascular hemodynamics can lead to multisystem organ complications. While there is no cure other than a bone marrow and blood transplant, there are several treatment options that can reduce symptoms and improve quality of life.

Assessing the efficacy of these treatments as well as the overall health of patients with SCD necessitates characterization of vascular health and hemodynamics. However, current clinical techniques for assessing vascular health require hospital visits, which are both time- and resource-intensive. Point-of-care methods to non-invasively characterize vascular health would be beneficial for optimizing treatments, as well as establishing a more complete assessment of vascular health.

Optical imaging and spectroscopy techniques are uniquely suited to enhance characterizations of vascular health in patients with SCD. Modalities such NIRS, LDF, and LSI can non-invasively quantify tissue oxygenation, blood flow, and hemoglobin concentration in the microvasculature. Additionally, over the last decade research has focused on developing handheld or wearable platforms, which would lessen the need for frequent hospital visits to perform health assessments. We are collaborating with Swee Lay Thein’s group to perform a series of optical measurements on a cohort of patients with SCD in order to characterize their vascular health and hemodynamics and compare their responses to a cohort of healthy volunteers. Additionally, we will work with Thein’s group to perform optical measurements on a cohort of SCD patients as they undergo various treatments, in order to characterize efficacy.

Additional Funding

• “Development of a Wearable Point of Care Monitoring Device for Pediatric Obstructive Sleep Apnea,” NICHD Director’s Award, NIH IRP

Publications


**Collaborators**

- Yama Akbari, MD, *University of California, Irvine, Irvine, CA*
- Ruth Benca, MD, PhD, *University of California, Irvine, Irvine, CA*
- Manfred Boehm, MD, *Laboratory of Cardiovascular Regenerative Medicine, NHLBI, Bethesda, MD*
- Matthew Brenner, MD, *School of Medicine, University of California, Irvine, Irvine, CA*
- Ashura Buckley, MD, *Office of the Clinical Director, NIMH, Bethesda, MD*
- Leighton Chan, MD, MPH, *Rehabilitation Medicine, Clinical Center, NIH, Bethesda, MD*
- Bernard Choi, PhD, *University of California, Irvine, Irvine, CA*
- Amir Gandjbakhche, PhD, *Section on Analytical and Functional Biophotonics, NICHD, Bethesda, MD*
- Ahmed Gharib, MD, *Biomedical and Metabolic Imaging Branch, NIDDK, Bethesda, MD*
- Sylvain Gioux, PhD, *Université de Strasbourg, Strasbourg, France*
- Michael Green, PhD, *University of California, Irvine, Irvine, CA*
- Ramy Khayat, MD, *School of Medicine, University of California, Irvine*
- Natasha Mesinkovska, MD, *University of California, Irvine, Irvine, CA*
- Thomas O’Sullivan, PhD, *University of California, Irvine, Irvine, CA*
- Thomas J. Pohida, MS, *Center for Information Technology, NIH, Bethesda, MD*
- Randall Pursley, *Center for Information Technology, NIH, Bethesda, MD*
- Kim Sehwan, PhD, *Dankook University, Yongin, South Korea*
- Swee Lay Thein, MB, FRCP, FRCPath, DSc, *Sickle Cell Branch, NHLBI, Bethesda, MD*

**Contact**

For more information, email *bruce.tromberg@nih.gov.*
The major focus of the Section is to understand how the elaborate networks of blood and lymphatic vessels arise during vertebrate development. Blood vessels supply every tissue and organ with oxygen, nutrients, and cellular and humoral factors. Lymphatic vessels drain fluids and macromolecules from the interstitial spaces of tissues, returning them to the blood circulation, and they play an important role in immune responses. Our studies on the formation of blood and lymphatic vessels are of great clinical interest because of the roles both types of vessels play in cancer and ischemia.

The zebrafish (*Danio rerio*), a small tropical freshwater fish, possesses a unique combination of features that make it particularly suitable for studying vessel formation. Zebrafish are genetically tractable vertebrates with externally developing, optically clear embryos, which are readily accessible for observation and experimental manipulation. Such features permit observation of every vessel in the living animal and simple, rapid screening for even subtle vascular-specific defects. Our current studies use genetic screening, experimental analysis, and imaging to examine cues directing vascular patterning and morphogenesis, regulation of vascular integrity, assembly of the lymphatic system, and the roles of novel vascular-associated cells.

In addition to our work on vessel development, we are pursuing studies on the role of epigenetics during early development, in particular how DNA methylation and other epigenetic mechanisms help coordinate cell, tissue, and organ specification and differentiation.

**Specification and patterning of developing blood vessels**

We are working to elucidate the cellular and molecular mechanisms responsible for the specification, patterning, and differentiation of blood vessels during development. Blood vessels are ubiquitous and vital components of vertebrate animals, innervating and supplying every tissue and organ with oxygen and nutrients. Many of the recent insights into mechanisms of blood vessel formation have come from studies in model organisms including the zebrafish. In zebrafish every blood vessel can be observed in living animals with high resolution.
FIGURE 1. The zebrafish vascular system

Confocal micro-angiogram of the vascular system of a 4½-day-old zebrafish larva labeled by injecting fluorescent microspheres. The transparency of zebrafish larvae makes it possible to use high-resolution optical imaging methods to visualize the entire vasculature in exquisite detail.

and simple, rapid screening can be accomplished for even subtle vascular-specific mutants (Figure 1). We are carrying out several related projects using the fish, which are described below.

NEW TOOLS FOR EXPERIMENTAL ANALYSIS OF VASCULAR DEVELOPMENT

We generate novel transgenic lines for visualizing different endothelial cell and perivascular cell types and for driving gene expression or performing molecular profiling of mRNAs and microRNAs in these cell populations.

GENETIC ANALYSIS OF VASCULAR DEVELOPMENT

We have identified many novel mutants affecting vascular development in our transgene-assisted forward-genetic screens and are currently characterizing the phenotypes and molecular basis for several of such mutants.

ANALYSIS OF VASCULAR SPECIFICATION, PATTERNING, AND MORPHOGENESIS

We are studying the development of several vascular beds, including the vasculature of the pectoral fin, the fish equivalent of the mammalian forelimb.

Regulation of vascular integrity

We are using the zebrafish to understand the cellular and molecular mechanisms responsible for proper vessel morphogenesis and for the generation and maintenance of vascular integrity. Disruption of vascular integrity is associated with hemorrhagic stroke, a severe
FIGURE 3. Novel perivascular cells on the zebrafish brain

Confocal micrograph of fluorescent granular perithelial cells (FGPs, green) adhering to the outside of meningeal blood vessels (red) on the brain of a Tg(mrc1a:egfp); Tg(kdr1:cherry) double-transgenic adult zebrafish. We recently showed that FGPs are unique endothelium-derived perivascular cells with unusual scavenging properties that are likely to be critical for brain homeostasis.

and debilitating form of stroke associated with high morbidity and mortality. Meningeal vascular dysfunction is also associated with neurocognitive deficits and neurodegenerative disease. Many of the recent insights into the molecular mechanisms regulating vascular integrity have come from studies in model organisms such as the zebrafish. We are pursuing several related projects.

GENES REGULATING VASCULAR INTEGRITY

We used forward-genetic screens to identify new zebrafish mutants that disrupt cranial vascular integrity in the zebrafish (Figure 2), using next-gen sequencing methods to accomplish higher throughput cloning of mutants. We already characterized the role of GDF6 (growth differentiation factor 6, also known as BMP13) in vascular integrity, demonstrating that the gene promotes maintenance of vascular integrity by suppressing excess VEGF (vascular endothelial growth factor) signaling. We are currently characterizing the molecular nature of defects in the regulatory protein RHOA (involved in cytoskeletal dynamics, transcription, cell cycle progression, and cell transformation), which result in vascular integrity defects.

ACQUISITION AND FUNCTION OF SUPPORTING VASCULAR SMOOTH MUSCLE CELLS

The vascular smooth cells (VSMC) that surround the endothelial tube play a critical role in regulating vascular tone and vascular integrity. We examined the early origins of the cells, how their interaction with endothelial tubes helps maintain the vascular basement membrane and restricts vessel diameter, and the molecular mechanisms underlying the arterial (versus venous) specific recruitment of VSMC.

VASCULATURE AND VASCULAR-ASSOCIATED CELLS IN THE MENINGES

The meninges are an external enveloping connective tissue that encases the brain, producing cerebrospinal fluid, acting as a cushion against trauma, nourishing the brain via nutrient circulation, and removing waste. Despite its importance, the cell types present in the meninges and the function and embryonic origins of the tissue are still not well understood. We recently discovered and characterized fluorescent granular perithelial cells (FGPs) in the zebrafish, a novel endothelium-derived perivascular cell population closely associated with meningeal blood vessels, which is likely to play a critical role in meningeal function (Figure 3). We are currently carrying out additional studies to understand the function of FGPs and other novel meningeal vascular-associated cell populations.
We generated new transgenic lines that permit direct, specific visualization of the developing lymphatic vasculature and are using sophisticated imaging of these transgenic animals to characterize lymphatic development (Figure 4).

We carried out forward-genetic ENU (\(N\)-ethyl-\(N\)-nitrosourea) mutagenesis screens using our lymphatic reporter transgenic lines to identify new lymphatic-specific mutants with defects in novel genes that play important roles in lymphatic development.

We are characterizing and studying novel microRNAs expressed in the lymphatic endothelium and how these small regulatory RNAs influence lymphatic gene expression and lymphatic development.

We are studying the formation of previously uncharacterized lymphatic vascular networks surrounding the zebrafish brain. Like similar brain lymphatic vessels recently discovered in the mammalian brain, the zebrafish vessels are likely to play critical roles in maintaining homeostasis and protecting the brain, and we are carrying out a detailed analysis of the development, form, and function of these critical vessels.

The results of our studies, combining the genetic and experimental tools available in the zebrafish with the ability to perform high-resolution microscopic imaging of developing vascular structures in living animals, will continue to lead to important new insights into the origins and growth of the lymphatic system and molecular mechanisms that are critical in lymphatic development and lymphatic pathologies.

**SECTION ON VERTEBRATE ORGANOGENESIS**

**FIGURE 4. Novel lymphatic vascular reporter**

Lateral view confocal image of the trunk of a 12 dpf (days post-fertilization) \(Tg(kdr:cherry); Tg(mrc1a:egfp)\) double-transgenic zebrafish with red fluorescent blood vessels and green fluorescent lymphatics. See Jung HM, et al. Development 2017;144:2070 for additional details.

**Specification and patterning of the lymphatic system**

The lymphatic system is a vascular system completely separate from the blood circulatory system and comprises an elaborate blind-ended tree of vessels that extensively innervate most of the body, emptying lymph fluid into the venous blood vascular system via several evolutionarily conserved drainage points. The lymphatic system is essential for immune responses, fluid homeostasis, and fat absorption, and is involved in many pathological processes, including tumor metastasis and lymphedema. However, progress in understanding the origins and early development of the system has been hampered by difficulties in observing lymphatic cells \textit{in vivo} and performing defined genetic and experimental manipulation of the lymphatic system in currently available model organisms. Our groundbreaking studies demonstrated that zebrafish possess a lymphatic system that shares many of the morphological, molecular, and functional characteristics of lymphatic vessels found in other vertebrates, providing a powerful model for the purpose of imaging and studying lymphatic development. We are currently pursuing further study of the formation of the lymphatic system through several ongoing projects.
The global burden of diabetes has risen dramatically, with projections that more than 600 million adults will be affected by 2030. Micro- and macrovascular complications in patients with diabetes are the major causes of cardiovascular mortality, renal failure, blindness, and non-traumatic amputations. Diabetes-related complications can emerge even many years after the blood sugar levels have been brought under control, a phenomenon known as “glycemic memory.” Although the cause of the phenomenon remains to be elucidated, epigenetic alterations in endothelial cells (ECs) may be responsible for the perdurance of diabetic vascular effects. We are using the zebrafish as an \textit{in vivo} model to examine whether short-term exposure to hyperglycemia results in persistent transcriptomic and epigenomic changes in endothelial cells, even after return to normoglycemic conditions. We identified several genes with significantly altered endothelial transcription and methylation levels during hyperglycemia that persist during the memory phase. We are currently carrying out further investigation of such “glycemic memory loci” using CRISPR genome editing and other methods. Unveiling the epigenetic and transcriptomic landscape of glycemic memory in ECs may lead to a better understanding of the mechanisms underlying diabetes-related vascular complications.

**EPIGENETIC REGULATION OF FAT AND MUSCLE DEVELOPMENT IN CAVEFISH**

In addition to eye and pigment loss and other adaptations, \textit{Astyanax} cavefish (Figure 5) have extreme and unusual metabolic adaptations that allow them to survive chronic and long-term food deprivation, including excess fat deposition, altered liver function, and resistance to metabolic disease. We hypothesize that, in a similar manner to loss of eyes, changes in epigenetic gene regulation may also underlie cavefish metabolic adaptations. We are using single-cell profiling to investigate differences in adipocytes and other cell types in the muscles (where in cavefish there are large amounts of fat stored) and livers of cavefish and surface fish. We are also performing whole-genome bisulfite sequencing and RNA-Seq from surface and cavefish muscles and livers to identify differentially expressed and methylated genes. We will follow up on these findings to elucidate how differential DNA methylation influences fat metabolism and obesity.

**UNCOVERING MOLECULAR MEDIATORS OF GLYCEMIC MEMORY IN DIABETIC VASCULOPATHY**

We are using the genetically and experimentally accessible zebrafish and Mexican tetra (\textit{Astyanax mexicanus}) models to uncover the molecular basis for organ- and tissue-specific epigenetic regulation during development in the following interrelated projects.

**Epigenetics of development**

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**FIGURE 5. Mexican tetra cave and surface fish**

The Mexican tetra \textit{Astyanax mexicanus} is a freshwater fish native to parts of southern Texas and eastern and central Mexico, which exists in both surface-dwelling (“surface morphs,” top right) and very closely related cave-dwelling (“cave morphs,” bottom left) populations. Cave morphs have a series of uniquely evolved adaptations including loss of eyes and pigment, dramatically altered metabolism, altered vascular function, and altered sleep regulation and behavior. Results from our laboratory suggest that altered DNA methylation and resulting coordinated changes in expression of large sets of genes have helped drive at least some of this rapid evolutionary change.

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FIGURE 6. An epigenetic silencing mutant in the zebrafish

Lateral views of the head and anterior trunk of a wild-type (top) and tissue-specific epigenetic silencing mutant (bottom) zebrafish. The mutant causes loss of epigenetic silencing specifically in the liver (red arrows), as visualized with a novel transgenic reporter line developed in our lab, which permits dynamic, tissue-specific visualization of epigenetic silencing in living animals.

to better identification of molecular targets and, potentially, to the design of personalized, epigenetic-based therapies to alleviate the enormous burden of diabetic vasculopathy.

FORWARD-GENETIC SCREEN FOR EPIGENETIC REGULATORY FACTORS

Genetic screens carried out in Drosophila and the nematode Caenorhabditis elegans have been highly successful in identifying genes regulating cell type–specific epigenetic gene regulation in invertebrates, but the molecular mechanisms involved in organ- and tissue-specific epigenetic regulation in vertebrates are still relatively unknown. We developed a novel zebrafish transgenic reporter line that allows us to monitor dynamic changes in epigenetic regulation in intact animals during development. Using the transgenic line, we are performing the first large-scale F3 genetic screen in a vertebrate to identify recessive mutants in regulators of epigenetic gene silencing or activation (Figure 6).

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- NICHD Intramural Research Fellowship (to L. Greenspan)

Publications


**Collaborators**
- Andreas Baxevanis, PhD, *Computational and Statistical Genomics Branch, NHGRI, Bethesda, MD*
- Harold Burgess, PhD, *Section on Behavioral Neurogenetics, NICHD, Bethesda, MD*
- George Davis, PhD, *University of Missouri-Columbia, Columbia, MO*
- Elisabetta Dejana, PhD, *The FIRC Institute of Molecular Oncology Foundation, Milan, Italy*
- Silvio Gutkind, PhD, *Oral and Pharyngeal Cancer Branch, NIDCR, Bethesda, MD*
- James Iben, PhD, *Molecular Genomics Laboratory, NICHD, Bethesda, MD*
- Sumio Isogai, PhD, *Iwate Medical University, Morioka, Japan*
- William R. Jeffery, PhD, *University of Maryland, College Park, MD*
- Paul Liu, MD, PhD, *Genetics and Molecular Biology Branch, NHGRI, Bethesda, MD*
- Richard Marais, MD, *Section on Molecular and Cell Biology, NICHD, Bethesda, MD*
- Yoh-suuke Mukouyama, PhD, *Laboratory of Stem Cell and Neuro-Vascular Biology, NHLBI, Bethesda, MD*
- Lisa M. Price, PhD, *Division of Developmental Biology, NICHD, Bethesda, MD*
- Radu V. Stan, MD, PhD, *Geisel School of Medicine at Dartmouth, Lebanon, NH*

**Contact**
For more information, email weinsteb@mail.nih.gov or visit https://www.nichd.nih.gov/research/atNICHD/Investigators/weinstein.
Studies on DNA Replication, Repair, and Mutagenesis in Eukaryotic and Prokaryotic Cells

Under optimal conditions, the fidelity of DNA replication is extremely high. Indeed, it is estimated that, on average, only one error occurs for every 10 billion bases replicated. However, given that living organisms are continually subjected to a variety of endogenous and exogenous DNA-damaging agents, optimal conditions rarely prevail in vivo. While all organisms have evolved elaborate repair pathways to deal with such damage, the pathways rarely operate with 100% efficiency. Thus, persisting DNA lesions are replicated, but with much lower fidelity than in undamaged DNA. Our aim is to understand the molecular mechanisms by which mutations are introduced into damaged DNA. The process, commonly referred to as trans-lesion DNA synthesis (TLS), is facilitated by one or more members of the Y-family of DNA polymerases, which are conserved from bacteria to humans. Based on phylogenetic relationships, Y-family polymerases may be broadly classified into five subfamilies: DinB–like (pol IV/pol kappa–like) proteins are ubiquitous and found in all domains of life; in contrast, the Rev1–like, Rad30A (pol eta)–like, and Rad30B (pol iota)–like polymerases are found only in eukaryotes; and the UmuC (polIV)–like polymerases only in prokaryotes. We continue to investigate TLS in all three domains of life: bacteria, archaea, and eukaryotes.

Prokaryotic studies

As part of an international scientific collaboration with Andrew Robinson, Myron Goodman, and Michael Cox, we investigated the role of *Escherichia coli* DNA polymerase IV (pol IV) in double strand break repair. To do so, Andrew Robinson’s group used live-cell single-molecule microscopy with fluorescently tagged pol IV and found that exposure to ciprofloxacin and trimethoprime antibiotics leads to the formation of double strand breaks in *E. coli* cells that strongly stimulate pol IV activity. Furthermore, the RecA recombinase and pol IV foci increase after antibiotic treatment and exhibit strong colocalization. Interestingly, the induction of the SOS response, the appearance of RecA foci, the appearance of pol IV foci, and RecA-pol IV colocalization, all depend on RecB function. We hypothesized that the positioning of pol IV foci likely reflects a physical interaction with nucleoprotein filaments denoted RecA* that was detected previously in vitro. Our observations therefore provided an in vivo substantiation of a direct role for pol IV in double strand break repair in cells treated with double strand break–inducing antibiotics [Reference 1].

Roger Woodgate, PhD, Head, Section on DNA Replication, Repair and Mutagenesis
Alexandra Vaisman, PhD, Interdisciplinary Scientist
John P. McDonald, PhD, Biologist
Mary McLenigan, BS, Chemist
Nicholas W. Ashton, PhD, Postdoctoral Visiting Fellow
Mallory R. Smith, PhD, Postdoctoral Intramural Research Training Award Fellow
Katherine Mnuskin, BS, Postbaccalaureate Intramural Research Training Award Fellow
Dominic R. Quiros, BS, Postbaccalaureate Intramural Research Training Award Fellow
Nicole Wilkinson, BS, Postbaccalaureate Intramural Research Training Award Fellow
Maya Kaplan, Stay-in-School Intramural Research Training Award Fellow
Eukaryotic studies
Maintaining the genomic integrity of cells is vital, as alterations to the genetic code can result in deregulation of cellular function, malignant transformation, or cell death. This can lead to a variety of disorders including neurological degeneration, premature aging, developmental defects and cancer. To prevent genetic alterations, cells employ a range of genome-stability pathways, which allow for the accurate metabolism of the DNA, as well as for any DNA errors or damage to be rapidly repaired. Post-translational modifications play an essential role in the signaling, activation and coordination of the genome stability pathways [References 2 & 3]. The reversible ubiquitination of proteins is one such essential modification. Ubiquitination is mediated by a cascade of E1, E2, and E3 ubiquitin enzymes, which covalently attach the 8.5 kDa ubiquitin protein onto a substrate molecule, while deubiquitinating enzymes (DUBs) can edit or remove ubiquitin modifications.

Human DNA polymerase iota (pol iota) was discovered by scientists in our lab two decades ago, yet its cellular function remains enigmatic [Reference 4]. As part of our ongoing research on pol iota, we previously reported that the enzyme is ubiquitinated at over 27 individual sites in the 715 amino acid protein. In collaborative studies with Irina Bezsonova, we have now identified Ubiquitin-Specific Protease 7 (USP7) as the enzyme that de-ubiquitinates pol iota. This is extremely interesting, as USP7 has recently emerged as a key regulator of ubiquitination in the genome stability pathways because of its extensive network of interacting partners and established roles in cell-cycle activation, immune responses, and DNA replication. USP7 is also deregulated in many cancer types, where deviations in USP7 protein levels are correlated with cancer progression.

USP7 contains of an N-terminal tumor necrosis receptor-associated factor (TRAF)–like domain, a catalytic domain, and five C-terminal ubiquitin-like domains (UBLs). While the catalytic domain mediates the enzymatic function of the protein, the TRAF-like and UBL domains are essential for substrate specificity and enzymatic activation. These functions are mediated by protein-binding sites, located on TRAF and the first and second UBL domain (UBL1–2). Interestingly, while all other characterized USP7 substrates bind to one or the other protein-binding sites, our studies with DNA polymerase iota revealed that a novel USP7 substrate interacts with both domains. Using biophysical approaches and mutational analysis, we characterized both interfaces and demonstrated that bipartite binding to both USP7 domains is required for efficient DNA polymerase iota de-ubiquitination. Taken together, our data established a new bipartite mode of USP7–substrate binding [Reference 5].

Publications
Collaborators

- Irina Bezsonova, PhD, University of Connecticut, Farmington, CT
- Anders R. Clausen, PhD, Göteborgs Universitet, Göteborg, Sweden
- Michael Cox, PhD, University of Wisconsin, Madison, WI
- Iwona Fijlakowska, PhD, Polish Academy of Sciences, Warsaw, Poland
- Myron F. Goodman, PhD, University of Southern California, Los Angeles, CA
- Karolina Makiela-Dzbenska, PhD, Polish Academy of Sciences, Warsaw, Poland
- Justyna McIntyre, PhD, Polish Academy of Sciences, Warsaw, Poland
- Andrew Robinson, PhD, University of Wollongong, Wollongong, Australia
- Ewa Sledziewska-Gojska, PhD, Polish Academy of Sciences, Warsaw, Poland
- Antoine Van Oijen, PhD, University of Wollongong, Wollongong, Australia
- Digby Warner, PhD, University of Cape Town, Cape Town, South Africa
- Wei Yang, PhD, Laboratory of Molecular Biology, NIDDK, Bethesda, MD

Contact
For more information, email woodgate@nih.gov or visit http://sdrrm.nichd.nih.gov.
Physiology, Psychology, and Genetics of Obesity

The prevalence of overweight and obesity in children and adults has greatly increased during the past 40 years; the alarming rise in body weight has likely occurred because the current environment affords easy access to calorie-dense foods and requires less voluntary energy expenditure. However, such an environment leads to obesity only in those individuals whose body weight–regulatory systems are not able to control body adiposity with sufficient precision in our high calorie/low activity environment, suggesting that there are subgroups in the U.S. with a uniquely high susceptibility to weight gain under the prevailing environmental conditions. Our primary goal is to elucidate the genetic underpinnings of the metabolic and behavioral endophenotypes that contribute to the development of obesity in children. Using our unique longitudinal cohorts of children at risk for adult obesity, who have undergone intensive metabolic and behavioral phenotyping, we examine genetic and phenotypic factors predictive of progression to adult obesity in children who are in the “pre-obese” state, allowing characterization of phenotypes unconfounded by the impact of obesity itself. Once they are identified as linked to obesity, we intensively study genetic variants that impair gene function. We expect that these approaches will improve our ability to predict which children are at greatest risk for obesity and its comorbid conditions and will lead to more targeted, etiology-based, “precision medicine” prevention and treatment strategies for pediatric obesity.

Genetic factors important for childhood body weight regulation

To identify gene variants affecting body composition, we have been examining polymorphisms in genes involved in the leptin signaling pathway. Such genes include the leptin receptor (LEPR), genes that appear to alter leptin receptor signal transduction such as those that are part of the BBSome (Bardet-Biedl syndrome [BBS]), a genetic condition characterized by obesity, retinal degeneration, polydactyly, hypogonadism, renal failure, and learning difficulties; the BBSome is an octomeric protein complex of BBS proteins, mutation in which cause BBS), and those encoding proopiomelanocortin (POMC), the melanocortin 3 receptor (MC3R), the melanocortin 4 receptor (MC4R), and brain-derived neurotrophic factor (BDNF).
We are currently studying a variant MC3R that is associated with adiposity in children and adults [Reference 1] and appears to have functional significance for MC3R signal transduction. Children and adults who were homozygous-variant for both C17A and G241A polymorphisms have significantly greater fat mass and higher plasma levels of insulin and leptin than unaffected or heterozygous children and appear to eat more at laboratory test meals (Figure 1). In vitro studies subsequently found that signal transduction and protein expression were significantly lower for the double mutant MC3R. In our ongoing studies we are attempting to understand the mechanisms by which these sequence alterations affect body weight. We therefore developed transgenic ‘knock-in’ mice expressing the human wild-type and human double-mutant MC3R. Using homozygous knock-in mouse models replacing murine Mc3r with wild-type human (MC3R<sup>hWT/hWT</sup>) and double-mutant (C17A+G241A) human (MC3R<sup>hDM/hDM</sup>) MC3R, we found that MC3R<sup>hDM/hDM</sup> have greater weight and fat mass (Figure 2), increased energy intake and feeding efficiency, but lower length and fat-free mass than MC3R<sup>hWT/hWT</sup>. MC3R<sup>hDM/hDM</sup> mice do not have increased adipose-tissue inflammatory cell infiltration or greater expression of inflammatory markers despite their greater fat mass. Serum adiponectin is increased in MC3R<sup>hDM/hDM</sup> mice and MC3R<sup>hDM/hDM</sup> human subjects (Figure 2). MC3R<sup>hDM/hDM</sup> bone- and adipose tissue-derived mesenchymal stem cells (MSCs) differentiate into adipocytes that accumulate more triglyceride than MC3R<sup>hWT/hWT</sup> MSCs. MC3R<sup>hDM/hDM</sup> thus impacts nutrient partitioning to generate increased adipose tissue, which appears metabolically healthy. The data confirm the importance of MC3R signaling in human metabolism and suggest a previously unrecognized role for the MC3R in adipose tissue development. Ongoing studies continue to improve our understanding of the phenotype of these mice. We are investigating a novel role for the MC3R in the regulation of hepatic autophagy, the role of MC3R in stem cell fate, and how variations in Mc3r may alter signaling of several downstream signaling pathways. Using tissue-specific knockout and reactivation models, we are also studying the importance of hepatic and adipose-tissue MC3R for whole body homeostasis.

**Physiology, metabolism, and psychology of childhood body-weight regulation**

Our studies are directed at understanding the physiological, psychological, and metabolic factors that place children at risk for undue weight gain. We study normal-weight children and adolescents, children who
already have obesity, and the children of parents with obesity who do not have obesity, in order to determine the factors that are most important for developing the complications of obesity in youth. We examine body composition, leptin concentration, metabolic rate, insulin sensitivity, glucose disposal, energy intake at buffet meals, and genetic factors believed to regulate metabolic rate and body composition. We also study psychological and behavioral factors, such as propensity to engage in binge-eating behavior (Figure 3), and sleep [Reference 3]. Children are being followed longitudinally into adulthood. In two protocols, we study actual food consumption of children during meals to elucidate differences in the calorie and macronutrient content of meals and the circulating hormones related to hunger and satiety in those who either endorse binge-eating behaviors or report no such behaviors. We found that eating in the absence of physiological hunger is a replicable trait that appears linked to obesity. We also investigated the role of sedentary behaviors, such as television watching, as a factor that alters metabolism. In a randomized, controlled, crossover trial (Figure 4), we found that glucose homeostasis was markedly improved in children with overweight or obesity who engaged in moderate activity for just three minutes every half hour, versus remaining sedentary.

As part of these studies, we examined how best to measure eating-related psychopathology, insulin sensitivity, changes in body composition, energy intake, and energy expenditure in children, and we studied the short- and long-term stability of the components of metabolic syndrome. We previously found that leptin is an important predictor of weight gain in children and identified children with hyperleptinemia and leptin receptor mutations. We also found that hyperleptinemia was out of proportion with body fat mass in children with psychological loss of control (LOC) over eating. Such data suggest the importance of leptin resistance as a factor stimulating weight gain, and they led to recent explorations of other syndromes associated with obesity that may cause dysregulation of leptin signaling, including WAGR, Bardet-Biedl [Reference 2], and Alström (characterized among other things by vision and hearing abnormalities, childhood obesity, and cardiomyopathy) syndromes. Current studies are directed at understanding additional genetic, physiological, and psychological factors that place children at risk for undue weight gain, including humoral factors, sleep [Reference 3], negative affective states such as depression and anxiety, weight-based teasing, alexithymia,
executive functioning [Reference 4], and LOC eating. Some recent initiatives have targeted insulin resistance in girls at high risk for type 2 diabetes because of obesity and a family history of diabetes.

Our evaluations concentrating on binge-eating behaviors in children suggest that such behaviors also are associated with adiposity in children and with abnormalities in metabolism. We found that binge-eating behaviors may predict future weight gain in children at risk for obesity. Thus, children reporting binge-eating behaviors such as loss of control over eating gained, on average, an additional 2.4 kg of weight per year compared with non-binge-eating children. Our data also suggest that children endorsing binge eating consume more energy during meals. Actual intake during buffet meals averaged 400 kcal more in children with binge eating, but despite their greater intake, such children reported shorter-lived satiety than children without binge-eating episodes. The ability to consume large quantities of palatable foods, especially when coupled with reduced subsequent satiety, may play a role in the greater weight gain found in binge-eating children.

Among cohorts of lean and obese youth, we demonstrated that youth with LOC eating had significantly higher serum leptin levels and are at greater risk for worsening of components of the metabolic syndrome than those without LOC episodes, even after adjusting for adiposity and other relevant covariates. Our data also suggest that anxiety symptoms may interact with LOC eating to become an important co-factor for excessive weight gain among children. These data also suggest that interventions targeting disordered eating behaviors may be useful in preventing excessive fat gain in children prone to obesity and have led to trials of preventative strategies related to binge eating.

**Treatment of obesity and the co-morbid conditions associated with obesity**

Given the rapid increase in the prevalence of obesity, the development of treatments for obesity in children and adults is urgently needed, yet current pharmacologic approaches are extremely limited for both children and adults. In several clinical protocols, we examined approaches for the prevention and treatment of excessive body weight. We completed a randomized controlled trial to investigate the mechanism by which metformin may affect the body weight of younger children who have hyperinsulinemia and are therefore at risk for later development of type 2 diabetes. Compared with placebo-treated children, those randomized to metformin reduced their BMI, BMI-Z score, and body fat mass to significantly greater extents. Serum
in younger children, finding good tolerability for such a program. A fourth study examined whether reducing depressive symptoms could ameliorate insulin resistance in adolescents at risk for type 2 diabetes. Among girls with greater (moderate) baseline depressive symptoms (N = 78), those in cognitive behavioral therapy (CBT) developed significantly lower two-hour insulin than those in HE. Additional metabolic benefits of CBT were seen for this subgroup in post hoc analyses of post-treatment to one-year change. An ongoing study based on lab data finding links between attentional biases to high-palatability foods in children with obesity examines whether adolescents’ attentional biases can be retrained.

We also initiated a translational trial studying the effects of modulating the leptin signaling pathway with the melanocortin agonist setmelanotide in Bardet Biedl syndrome patients with proximal signaling defects [Reference 2]. Most recently, we initiated another study of specific pharmacotherapy for patients with the Prader-Willi syndrome using diazoxide. These latest trials are examples of precision medicine approaches to treat obesity. We also recently completed a novel randomized-controlled pilot trial of colchicine to ameliorate the inflammation of obesity and thus improve its complications [Reference 5]. Adults with obesity and metabolic syndrome, but who did not have diabetes, were randomized to colchicine 0.6 mg or placebo capsules twice daily for three months. Compared with placebo, colchicine significantly reduced C-reactive protein and erythrocyte sedimentation rate (Figure 5). The significant changes in HOMA-IR, fasting insulin, and glucose effectiveness suggested metabolic improvements in the colchicine versus placebo group. The results suggest a larger, adequately powered study should be conducted to determine whether colchicine improves insulin resistance and other measures of metabolic health in at-risk individuals.
FIGURE 5. Effects of colchicine on inflammatory and metabolic measures

Metabolic and inflammatory changes after three months of study medication in participants randomized to colchicine (N=21) or placebo (N=19).

A. Insulin sensitivity (SI).
B. Fasting glucose.
C. Fasting insulin.
D. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR).
E. High sensitivity C-reactive protein (hsCRP).
F. Erythrocyte sedimentation rate (ESR).
G. White blood cell count (WBC).
H. Neutrophil count.
I. Platelet count. Data are presented as mean ± SEM.

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Publications

Collaborators

- David Allison, PhD, School of Public Health, Indiana University, Bloomington, IN
- Jeffrey Baron, MD, Section on Growth and Development, NICHD, Bethesda, MD
- Karen Berman, PhD, Section on Integrative Neuroimaging, NIMH, Bethesda, MD
- Kong Chen, PhD, Clinical Endocrinology Branch, NIDDK, Bethesda, MD
- Ross Crosby, PhD, Director of Biomedical Statistics, Neuropsychiatric Research Institute, Fargo, ND
- Joel Elmquist, DVM, PhD, UT Southwestern Medical Center, Dallas, TX
- I. Sadaf Farooqi, MD, Cambridge Institute for Medical Research, Cambridge, United Kingdom
- Oksana Gavrilova, PhD, Mouse Metabolism Core Laboratory, NIDDK, Bethesda, MD
- Joan C. Han, MD, Le Bonheur Children’s Hospital, Memphis, TN
- Robert Haws, MD, Marshfield Clinic Research Institute, Marshfield, WI
- Steven B. Heymsfield, MD, Pennington Biomedical Research Center, Baton Rouge, LA
- Michael Jensen, MD, Mayo Clinic, Rochester, MN
- Sergey Leikin, PhD, Section on Physical Biochemistry, NICHD, Bethesda, MD
- Jennifer Lippincott-Schwartz, PhD, HHMI Janelia Research Campus, Ashburn, VA
- Cara Olsen, PhD, Uniformed Services University of the Health Sciences, Bethesda, MD
- Vipul Periwal, PhD, Laboratory of Biological Modeling, NIDDK, Bethesda, MD
- Daniel Pine, MD, Section on Development and Affective Neuroscience, NIMH, Bethesda, MD
- Douglas Rosing, MD, Cardiac Consultation Service, NHLBI, Bethesda, MD
- Peter J. Schmidt, MD, Section on Behavioral Endocrinology, NIMH, Bethesda, MD
- Lauren B. Shomaker, PhD, University of Colorado, Boulder, CO
- Eric Stice, PhD, Oregon Research Institute, Eugene, OR
- Marian Tanofsky-Kraff, PhD, Uniformed Services University of the Health Sciences, Bethesda, MD
- B. Timothy Walsh, PhD, Columbia University College of Physicians and Surgeons, New York, NY
- Denise E. Wilfley, PhD, Washington University School of Medicine, St. Louis, MO
- Joshua Zimmerberg, MD, PhD, Section on Integrative Biophysics, NICHD, Bethesda, MD

Contact

For more information, email yanovskj@mail.nih.gov or visit http://sgo.nichd.nih.gov.
The Biophysics of Protein–Lipid Interactions in Influenza and Coronavirus, Malaria, and Muscular Dystrophy

Eukaryotic life must create the many shapes and sizes of the system of internal membranes and organelles that inhabit the variety of cells in nature. Such membranes must remodel in order for cells to repair damaged plasmalemma and deal with infectious agents such as viruses and parasites. Such basic membrane mechanisms must be highly regulated and highly organized in various hierarchies in space and time to allow the organism to thrive despite environmental challenges, genetic instability, unpredictable food supply, and physical trauma. We are using our expertise and the techniques that we perfected over the years to address several different biological problems that have in common the underlying regulation or disturbance of protein/lipid interactions. Thus, the overall goal of this project is to determine the physico-chemical mechanisms of membrane remodeling in cells. This year, we focused on our continued research on the physiology of deadly viruses and parasites by shifting most resources towards the COVID-19 pandemic, in areas closest to our expertise:

1) There is great urgency to produce an effective vaccine against COVID-19, not only effective in aging and vulnerable populations, i.e., those more at risk to COVID-19 mortality, but also for early protection from multi-system inflammatory changes in children. Many of the best modern vaccines (e.g., HPV) have been produced using virus-like particles (VLP). Coronavirus presents unique problems in post-translational processing of the Spike (S) protein; our aim is to create a VLP vaccine candidate that recapitulates the native SARS-COV-2 replication cycle of mammalian cellular production and purify pauci-proteinaceous particles (PPP) to acceptable homogeneity for FDA approval as a vaccine candidate.

2) Survival of the SARS-COV-2 virus particle for person-to-person transmission and its ability to fuse with the host cell membrane are highly dependent on the viral envelope lipid composition. Determining the lipid composition and physical properties of attenuated virus and VLP will permit the study of membrane stability and fusogenicity under physiological conditions. Our goal is to give the scientific community a VLP to efficiently study virus infection in widely available non-BSL2 (biosafety-level 2) laboratory space.
3) Anti-viral therapeutic strategies reducing the impact of the SARS-COV-2 virus should not lag behind vaccine development. The crucial stage of infection is the moment when the viral genome first enters the cytoplasmic space to begin infection, i.e., the fusion of the viral and endosomal membrane of a cell. Merging of two membrane-enclosed compartments is a ubiquitous event in biology and is crucial to fertilization, exocytosis, bone and muscle development, intracellular trafficking, and viral infection. The underlying biophysical determinants in these diverse processes are the same: the fusion and leakage of the mostly phospholipid bilayers that comprise cell membranes. We are working now to incorporate a reporter for the membrane fusion of infection within the COVID-19 VLP described above, which NCATS has agreed to study for antivirals in its medium- and high-throughput screens.

4) We are equally committed to a deep understanding of the mechanism by which the Spike proteins of SARS-COV-2, HIV, and influenza work, so as to design drugs against their action. This year, our goal was to model the reason for cooperation between more than one Spike trimer to achieve the fusion of infection.

5) In 2018, there were about 228 million cases of malaria worldwide, with about 405,000 malaria deaths. Children aged under five are the most vulnerable group affected by malaria; in 2018, they accounted for 67% (272,000) of global malaria deaths. Our goal in studying malaria is to develop new antimalarial drugs. This year, we defined a new intercellular contact for cells that dwell within other cells: the host-parasite interface (HPI), which may be a drug target.

Exocytotic fusion, compensated by endocytotic fission, are the essence of complex membrane dynamics in living cells. Fusion and fission are key elements of synapses and other dynamic cellular trafficking networks, and are instants when organelles gain or lose their identities. Without exocytosis, the insulin-sensitive glucose transporter would never reach the plasma membrane of a muscle or fat cell, nor would inflammatory cells respond and kill neoplastic or pathogenic invaders, or deal with sick cells. Our Section's earliest work concentrated on model exocytosis systems, the physical properties and theoretical pathways required for exocytosis, and the discovery that tension spreads headgroups for hemifusion, then pulls open fusion pores to allow coalescence of adherent bilayers. However, while enabling us to focus on basic membrane biophysical properties and to help develop a theoretical framework for understanding membrane interactions, model systems are a simplification that ignored the important roles of other cellular components. Last year we reported on lipids that abound in cellular functions known as the phosphoinositides. This year, we focused on the membrane mechanics that govern membrane stability and energetics.

New model for direct physical action of viral fusion proteins

Enveloped viruses include the most dangerous human and animal pathogens, in particular coronavirus, influenza virus, and human immunodeficiency virus (HIV). For these viruses, receptor binding and entry are accomplished by a single viral envelope protein (termed the fusion protein), the structural changes in which trigger the remodeling and merger of the viral and target cellular membranes. The number of fusion proteins required for fusion activity is still under debate, and several studies report this value to range from 1 to 9 for type I fusion proteins. We studied the earliest stage of viral fusion based on the continuum theory of membrane elasticity. We demonstrated that membrane deformations induced by the oblique insertion of amphipathic fusion peptides mediate the lateral interaction of the peptides and drive them to form into a symmetric fusion rosette. The pulling force produced by the structural rearrangements of the fusion protein...
ectodomains gives additional torque, which deforms the membrane and additionally stabilizes the symmetric fusion rosette, thus allowing a reduction in the number of fusion peptides needed for fusion. The findings can resolve the large range of published cooperativity indices for HIV, influenza, and other type I fusion proteins.

Two possible mechanisms of fusion protein action have been traditionally proposed. One is based on the assumption that the incorporated fusion peptides first of all modify the elastic properties of the target membrane, i.e., its spontaneous curvature. It is thought that the altered spontaneous curvature in the ring-like zone of the fusion rosette might be responsible for the formation of the bulge on the target membrane; the fusing membranes come into close contact at the top of the bulge, which substantially facilitates the merger of membranes. According to the second mechanism, instead of modifying the target membrane, the fusion proteins induce bending torques, causing the formation of bulges and generating pulling forces, thus directly and mechanically bringing two merging membranes into close contact. Based on the results of the present work, we can argue that the second mechanism provides the formation of highly symmetric fusion rosette owing to both tilting of the fusion peptides and pulling them out of the plane of the target membrane, thus ensuring cooperation of the mechanical efforts of fusion proteins. By contrast, the first mechanism uses the symmetry of the fusion rosette to explain the formation of the bulges on fusing membranes, rather than providing an explanation of the symmetry origin. We conclude that the direct mechanical activity of fusion proteins should drive the merger of membranes more reliably than local modification of the elastic properties of the target membrane by the incorporated fusion peptides. This activity could result from the concerted action of different subunits of fusion protein, placing the bound cell receptor molecule as an active player in the scene. The latter assumption could be the answer to the differences in the cooperativity of fusion proteins of influenza and HIV: we would need 39 pH–activated hemagglutinin trimers for effective fusion, while a single receptor-bound gp120/gp41 trimer of HIV would be sufficient.

The interface between the cytoplasm of the protozoan parasite and the cytoplasm of the host cell

For the malaria parasite, the host-parasite Interface (HPI) is an interface between the cytoplasm of the protozoan parasite and the cytoplasm of the human red blood cell. Rather than a simple bag within a bag, the HPI has structural complexity. First, it is formed by two membranes. We discovered that the space between the two membranes varies considerably, such that there are frequent sites of very close membrane contact. The sites of close membrane contact coincide with the location of PfNCR1, a lipid transporter, which allows direct lipid transport from membrane to membrane. The sites of contact exclude EXP2, a nutrient channel. Segregation of the two transporters by the contact sites may be a general mechanism to separate transport systems. We expect that the fundamental conceptual shift, i.e., to consider the HPI as a double membrane, domain-defined structure will generalize to other parasites. Furthermore, the HPI is likely to contain the kind of critical parasite-encoded proteins, such as PfNCR1 and EXP2, against which anti-malarial drugs can be made.

The malaria parasite interfaces with its host erythrocyte (RBC) using a unique organelle, the parasitophorous vacuole (PV). The mechanisms by which its limiting membrane, the parasitophorous vacuolar membrane (PVM), collaborates with the parasite plasma membrane (PPM) to support the transport of proteins, lipids, nutrients, and metabolites between the cytoplasm of the parasite and the cytoplasm of the RBC are obscure. We demonstrated that the structure of the PV is characterized by micrometer-sized regions of especially close apposition between the PVM and the PPM. To determine whether such contact sites are involved in any kind of transport, we localized the PVM nutrient-permeable and protein-export channel EXP2, as well as the PPM lipid
transporter PfNCR1. We found that EXP2 is excluded from, but that the PfNCR1 is included within, the regions of close apposition. We conclude that the host-parasite interface is structured to segregate such transporters of hydrophilic and hydrophobic substrates.

**Applying the quadratic energy functional monolayer-wise allows a generalization of the linear theory of membrane elasticity.**

Living cells are open non-equilibrium systems. To exist, a cell requires precisely controlled maintenance of gradients in the chemical potential between the extracellular environment, the cytoplasm, and the lumen of organelles, of many constituents. The amphiphilic nature of lipid molecules, self-assembling into lipid bilayers, provides an extremely low permeability barrier to both electrolytes and large nonelectrolytes. For the processes of life, a continuous exchange of matter must occur across all membranes. For example, uptake of large molecules and compounds from the outside occurs via endocytosis, phagocytosis, and macro- and micro-pinocytosis, secretion occurs via exocytosis, and intracellular protein trafficking via transport vesicles between endoplasmic reticulum, Golgi apparatus, endosomes, and lysosomes. The movement of such membrane-bound cargo dictates membrane recycling, otherwise cells and organelles would be incapable of maintaining their volumes and shapes. Such ubiquitous and multifarious events, plus the accommodation in lipid bilayers of membrane proteins and transient pores, all require the lipid bilayer to change its topology. Physical models of such topological changes, essential to life, must take into account the energy of membrane deformations within the framework of an adequate theory of elasticity.

The theory of elasticity of lipid membranes is used widely to describe processes of cell membrane remodeling. Classically, the functional of a membranes elastic energy is derived under assumption of small deformations; the membrane is considered as an infinitely thin film. The functional is quadratic on membrane surface curvature, with half of the splay modulus as its proportionality coefficient; it is generally applicable for small deformations only. Any validity of this functional for the regime of strong deformations should be verified experimentally. Recently, research using molecular dynamics simulations challenged the validity of this classic, linear model, i.e., the constancy of the splay modulus for strongly bent membranes. We demonstrated that the quadratic energy functional can still be applied for calculation of the elastic energy of strongly deformed membranes without introducing higher-order terms with additional elastic moduli, but only if applied separately for each lipid monolayer. For cylindrical membranes, both classic and monolayer-wise models yield equally accurate results. For cylindrical deformations we showed experimentally that the elastic energy of lipid monolayers is additive: a low molecular-weight solvent leads to an approximately twofold reduction in the membrane bending stiffness. Accumulation of solvent molecules in the inner monolayer of a membrane cylinder can explain the results, as the solvent partially prevents lipid molecules from splaying there. Thus, the linear theory of elasticity can be expanded through the range of weak to strong deformations. Its simplicity and physical transparency describe various membrane phenomena.

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Publications


Collaborators

- Sergei Akimov, PhD, Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia
- Pasha Bashkirov, PhD, Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia
- Oleg Batishchev, PhD, A.N. Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia
- Josh Beck, PhD, Iowa State University, Ames, IA
- Michael J. Blackman, PhD, The Francis Crick Institute and London School of Hygiene & Tropical Medicine, London, United Kingdom
- Nikki Curthoys, PhD, University of Maine, Orono, ME
- Andrew Demidowich, MD, PhD, Section on Growth and Obesity, NICHD, Bethesda, MD
- Vadim Frolov, PhD, Universidad del País Vasco, Bilbao, Spain
- Timur Galimzyanov, PhD, Frumkin Institute of Physical Chemistry and Electrochemistry, Russian Academy of Sciences, Moscow, Russia
- Daniel Goldberg, MD, PhD, Washington University St. Louis, St. Louis, MO
- Samuel T. Hess, PhD, University of Maine, Orono, ME
- Ron W. Holz, MD, PhD, University of Michigan Medical School, Ann Arbor, MI
- Mary Kraft, PhD, University of Illinois at Urbana-Champaign, Urbana, IL
- Louis H. Miller, MD, Laboratory of Malaria & Vector Research, NIAID, Bethesda, MD
- Richard Pastor, PhD, Laboratory of Membrane Biophysics, NHLBI, Bethesda, MD
- Thomas S. Reese, MD, Laboratory of Neurobiology, NINDS, Bethesda, MD
- Anna Shnyrova, PhD, Universidad del País Vasco, Bilbao, Spain
- Tobias Spielmann, PhD, Bernhard-Nocht-Institut für Tropenmedizin, Hamburg, Germany
- Peter K. Weber, PhD, Lawrence Livermore National Laboratory, Livermore, CA
• Jack Yanovski, MD, PhD, Section on Growth and Obesity, NICHD, Bethesda, MD

Contact
For more information, email Joshua.Zimmerberg@nih.gov or visit https://irp.nih.gov/pi/joshua-zimmerberg.
Dorsal view of the head of a 25-day-old Tg(mrc1a:egfp)\textsuperscript{y251}, Tg(Ola.Sp7:mCherry-Eco.NfsB)\textsuperscript{pd46} double transgenic zebrafish showing lymphatic vessels in orange and scales and bone in blue. The image was generated by Dan Castranova of the Weinstein lab.