National Cancer Institute

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CCR connections

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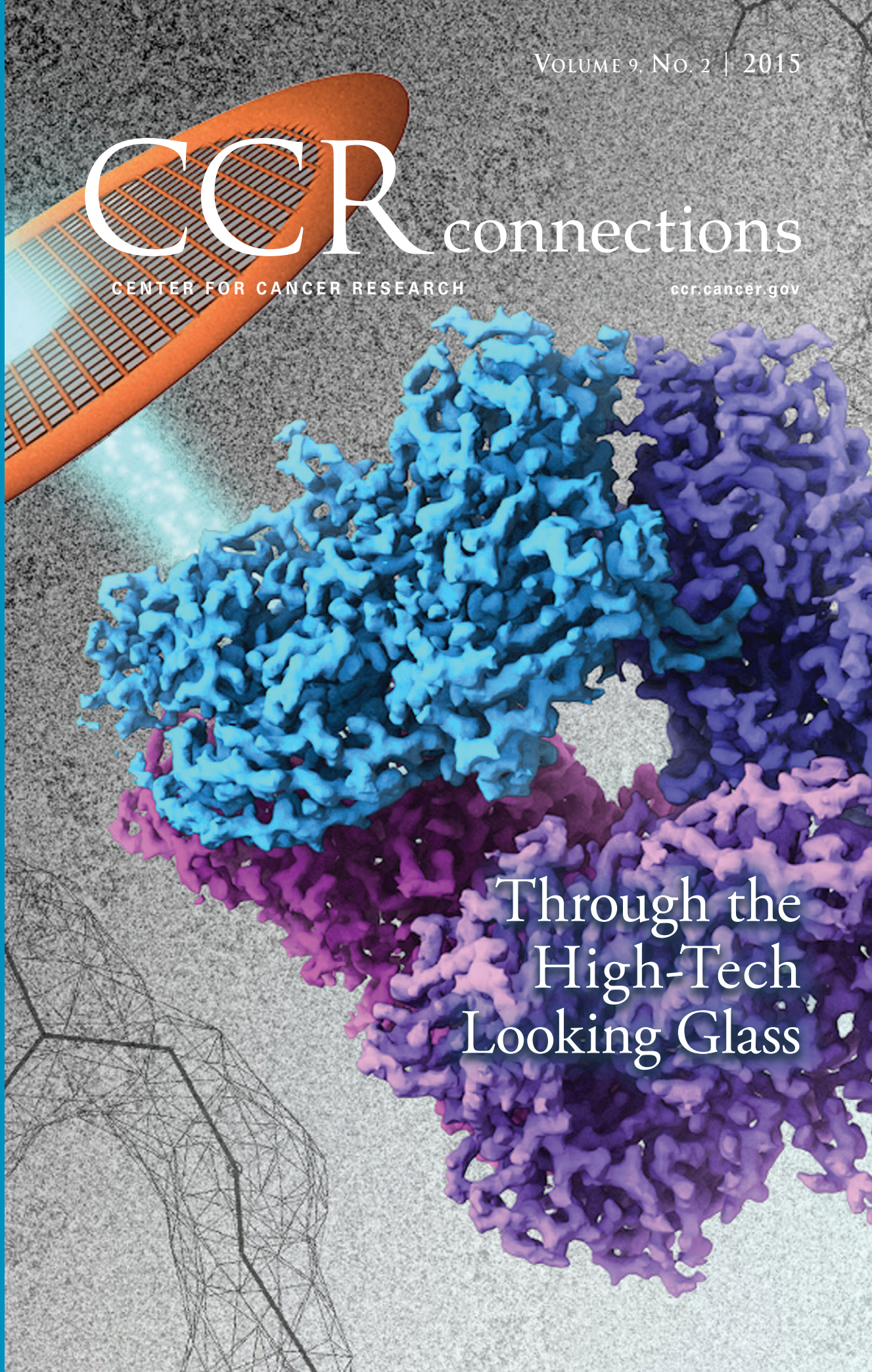

Through the High-Tech Looking Glass

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES

National Institutes of Health

About the Cover: Density map of the enzyme β-galactosidase determined by cryo-electron microscopy at 2.2 Å resolution (Bartesaghi, Merk et al., Science [2015]). The foreground shows the overall shape of the molecule (right), an electron beam striking a specimen grid (upper left), and the atomic contours of amino acids in the structure (lower left). The grey background shows a region from a representative raw cryo-EM image recorded in the electron microscope. *(Image: V. Falconieri and S. Subramaniam, CCR)*

We invite your comments and suggestions about CCR connections.

Please email your feedback to tellccr@mail.nih.gov.

Center for Cancer Research

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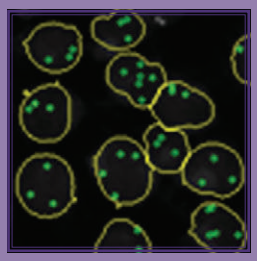

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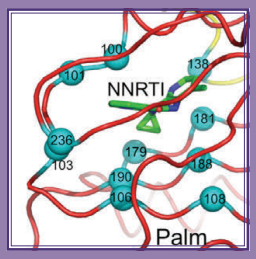

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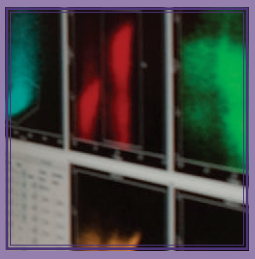

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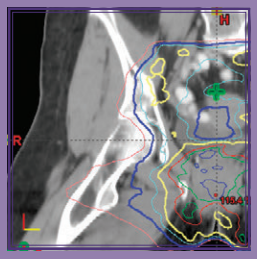

Radiation Therapy in the Modern World

NATIONAL CANCER INSTITUTE
Center for Cancer Research

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The mission of CCR is:

To inform and empower the entire cancer research community by making breakthrough discoveries in basic and clinical cancer research and by developing them into novel therapeutic interventions for adults and children afflicted with cancer or infected with HIV.

http://home.ccr.cancer.gov/connections

Synergies in Research

In 1981, the NCI intramural program enrolled its first patient with AIDS. Given our expertise in epidemiology, cancer, retroviruses, cell biology, immunology, and drug development, our responsibility in the face of this public health crisis seemed obvious. Indeed, NCI investigators were instrumental in such seminal milestones as discovering HIV, proving HIV as the causal agent of AIDS, developing the first blood test for HIV, describing the first antibodies to neutralize HIV, and developing the first active drug, AZT, for HIV/AIDS therapy.

Today, HIV/AIDS is no longer an automatic death sentence, but is a manageable chronic disease in much of the world. Although the immediate crisis has abated, CCR continues its commitment to HIV/AIDS research. The insights we gain from studying this virus and its impact on human cell physiology not only benefit those directly affected by HIV/AIDS, but also often strengthen our understanding of cancer.

As we learn in "Vaccines 2.0," Jay A. Berzofsky, M.D., Ph.D., Chief of CCR's Vaccine Branch, brings together research in both HIV and cancer within the branch as a whole and within his own laboratory. Berzofsky and his colleagues described the first T-cell epitopes of HIV; while his laboratory continues to focus on the immune response to HIV, it has also made fundamental advances in cancer immunotherapies. As Berzofsky points out, both HIV and cancer cause chronic disease that escapes the immune system through mutation. Both could benefit from therapeutic vaccines, which he and his team have been developing from cellular and animal models through to clinical trials.

Stephen Hughes, Ph.D., Chief of CCR's Retroviral Replication Laboratory, is internationally recognized for his work on essential enzymes in the HIV life cycle. He and his colleagues described some of the first structures of HIV-1 protease and reverse transcriptase. His work on retroviruses, however, began when he was a Postdoctoral Fellow with Harold Varmus, M.D., and Michael Bishop, M.D., who subsequently won the Nobel Prize in Medicine for their discovery of the cellular origin of retroviral oncogenes. And, as we learn in "Viral Activity," Hughes' work today has brought him back to the role that viral integration plays in abnormal cellular proliferation.

Featured in "Through the High-Tech Looking Glass," Sriram Subramaniam, Ph.D., Senior Investigator in CCR's Laboratory of Cell Biology, has developed techniques in high-resolution electron microscopy that bridge the domains of structural and cell biology. His work gives him unique access to the architectures of both human immunodeficiency viruses and cancer cells.

Young investigators are also drawn to the interface of virology and cancer biology. Joanna Sztuba-Solinska, Ph.D., who is currently a Research Fellow with Stuart LeGrice, Ph.D., Senior Investigator in CCR's Basic Research Laboratory, came to CCR in order to study the evolution of RNA viruses. Her work on the functional implications of complex RNA structures currently includes the cancer-causing Kaposi sarcoma herpesvirus, in addition to Ebola virus, Dengue virus, and other global health concerns.

(Photo: R. Baer)

Lee J. Helman, M.D., Acting CCR Director and CCR Scientific Director for Clinical Research

CCR's commitment to basic and clinical research that will lead to novel therapeutic interventions for both cancer and HIV is, thus, much more than a two-pronged response to distinct public health crises. It uniquely brings together two research agendas, which explore common biological mechanisms and insights, to synergistically advance our knowledge of, and ability to, respond to emerging issues related to cancer and HIV/AIDS.

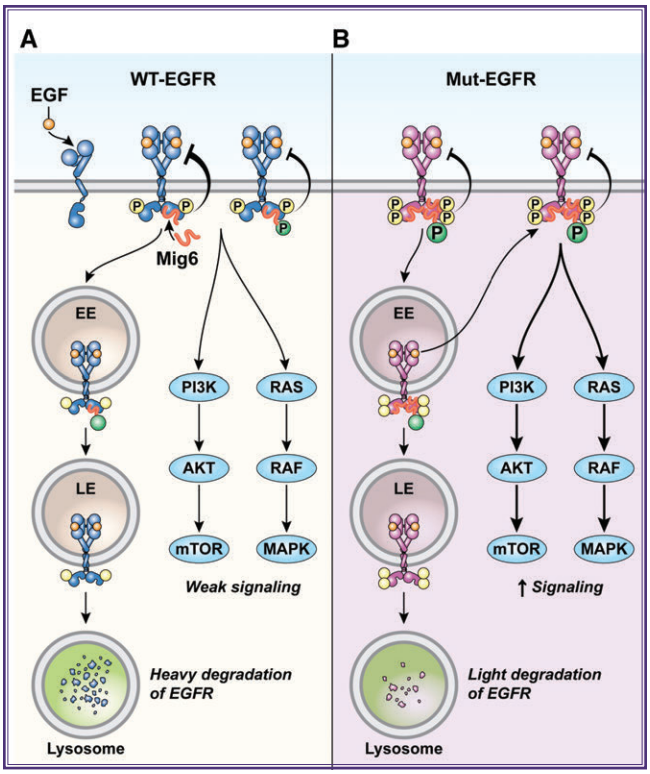
A Brake for Cancer

MIG6 suppresses tumor initiation and progression driven by mutant EGFR.

A common molecular driver of lung cancer, somatic mutations in the epidermal growth factor receptor (EGFR) can be differentially targeted with tyrosine kinase inhibitors (TKIs) such as gefitinib (Iressa) or erlotinib (Tarceva). Patients with such mutations in EGFR respond dramatically to these inhibitors, but all develop acquired drug resistance and eventually progress.

Udayan Guha, M.D., Ph.D., Investigator in CCR's Thoracic and Gastrointestinal Oncology Branch, and his colleagues have been studying the signaling pathways downstream of both wild-type and mutant EGFR activation, with the goal of contributing to more effective therapies. By using stable heavy isotope–labeled amino acids to tag the complete proteome, the team could quantify proteins that were highly phosphorylated in human bronchial epithelial cells expressing mutant EGFRs. As recently published in *Cancer Discovery*, they followed up on one such protein—MIG6—to uncover its role as a tumor suppressor.

MIG6 is known to regulate wild-type EGFR and other related receptors through a negative feedback process involving both increased degradation of the receptor and dampening of its tyrosine kinase activity. By generating a series of genetically modified mice, in which mice carrying 0, 1, or 2 copies of *Mig6* were also engineered to carry either of the two different inducible EGFR variants associated with lung cancer, the team found that mice lacking two copies of *Mig6* had accelerated lung tumor formation.



(Figure: U. Guha, CCR)

Mutations in EGFR alter its negative feedback interaction with MIG6.

To explore the mechanism of the interaction between MIG6 and mutant EGFR, the researchers returned to proteomics and they discovered that MIG6 is constitutively phosphorylated on one or two adjacent tyrosine residues (Y394/Y395) in both mouse and human lung adenocarcinoma cells expressing mutant EGFRs. Moreover, drug sensitivity to TKIs was correlated with this MIG6 phosphorylation, suggesting that it was important to the efficacy of mutant EGFR in promoting tumor growth.

In cell culture, the researchers found that Y394/Y395 phosphorylation strengthened the interaction between MIG6 and mutant EGFR. Unlike wild-type EGFR, whose degradation is enhanced by MIG6, mutant EGFR was stabilized by Y394/Y395 phosphorylation of MIG6. Based on these data, the researchers propose a model that contrasts the interaction of wild-type and mutant EGFR with MIG6. In wild-type EGFR, MIG6 acts as a negative feedback regulator of EGFR; once activated, the EGFR phosphorylates MIG6, which in turn inhibits the strength of downstream signaling through PI3K and RAS pathways, as well as promotes lysosomal degradation of the EGFR complex. However, the mutant EGFR hyperphosphorylates MIG6, dampening, but not completely abolishing, its ability to downregulate EGFR signaling.

"Our data provide strong preclinical evidence that MIG6 is a potent tumor suppressor in mutant EGFR-driven lung cancer," said Guha. "However, we will need prospective biomarker-validation studies to establish the role of MIG6 expression or phosphorylation in the overall prognosis of patients with and without EGFR mutations."

To learn more about Dr. Guha's research, please visit his CCR website at http://go.usa.gov/3uAqG.

The Power of Families

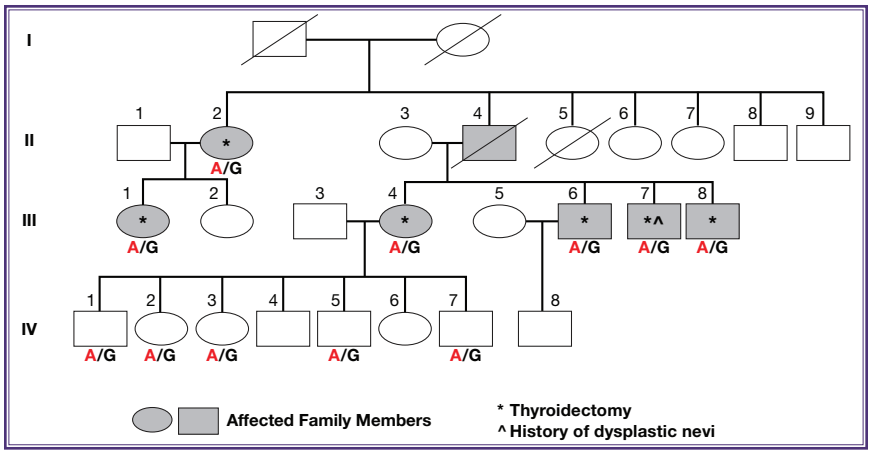
Genomic analysis of a family with thyroid cancer identifies HABP2 *as a tumor suppressor gene.*

Many diseases that are seemingly sporadic in the population at large cluster in certain families. Where genetic linkage can be established, researchers have an opportunity to discover specific DNA mutations that might give insight into not only the familial forms of the disease, but also the more widespread pathological mechanisms.

In 2010, Electron Kebebew, M.D., Chief of CCR's Endocrine Oncology Branch, and his colleagues began enrolling families in a clinical protocol to identify susceptibility genes for nonmedullary thyroid cancer (NMTC), the predominant form of thyroid cancer in the U.S. A family was referred to the NIH in which two of seven children and one member of the subsequent generation had been diagnosed with the disease. Ultrasound screening then revealed thyroid neoplasms in four other members of the third generation.

The researchers sequenced DNA from the blood of all affected family members and from unaffected spouses, using whole-exome sequencing to comprehensively search for mutations in the DNA that code for proteins. They discovered and validated a single-nucleotide substitution that was present in all seven affected family members: a substitution of adenine to guanine in the *HABP2* gene (G534E), which predicted a single amino acid change in the HABP2 protein. HABP2 is a serine protease found in the plasma, which cleaves fibrinogen and degrades the extracellular matrix. Molecular modeling of the amino acid change identified by the team revealed a disruption to the active site of the enzyme.

A single copy of the variant allele was present in the blood and tumor tissue of affected family members, which was consistent with the autosomal dominant pattern of the disease. Expression of the gene and protein was increased selectively in the tumor tissue. By surveying other cancer-associated mutations, rearrangements, and copy number variations, the researchers could find no evidence for additional genetic changes that might contribute to tumor formation.



(Figure: E. Kebebew, CCR)

A single family affected by nonmedullary thyroid cancer. Squares = males, Circles = females, Shading = affected members

Moving to cell lines, the researchers performed a series of experiments to study how altering the levels of wild-type and mutant HAPB2 affected cancerous phenotypes. Knocking down the wild-type form of the gene increased colony formation and cellular migration, while stable overexpression reduced these characteristics. Transient overexpression of the G534E variant, however, substantially increased the cancerous phenotype, even in the presence of wild-type HAPB2, suggesting that tumor suppression can indeed be disrupted by a single dominant variant.

Kebebew and his colleagues took advantage of The Cancer Genome Atlas (TCGA) to look for the presence of the G534E variant in more than 400 patients with nonmedullary thyroid cancer. They found the variant in 4.7 percent of patients, as compared with its presence in only 0.7 percent of a random sampling from a multiethnic population database. This suggests the presence of other cases of familial NMTC. The group's findings were published in the July 30, 2015, issue of the *New England Journal of Medicine.*

"Next, we need to consider how to handle screening for this variant," said Kebebew. "Should carriers of the variant undergo preventative thyroidectomy? Should carriers in the general population be screened for thyroid neoplasms? Does the variant confer a predisposition to other cancers?" These and many more questions on the role of this gene in cancer initiation and progression await further research.

To learn more about Dr. Kebebew's research, please visit his CCR webpage at *http://1.usa.gov/1PWo4OT.*

Shaping the Future of Cancer Research

CCR supports several programs to foster the next generation of biomedical scientists.

Twenty-five years ago, NCI administrator Gordon Cecil came to Howard Young, Ph.D., now a Senior Investigator in CCR's Cancer and Inflammation Program, with an idea to give local high school students first-hand exposure to scientific research at NCI at Frederick. From that conversation, the Werner H. Kirsten Student Intern Program was born, and nearly 1,000 students have gone through the program since that time. Seventy students make up the current class of interns.

"We started slowly," said Young. "The first year, we selected six students from an applicant pool of ten. Each year, we learned from what we had done before and modified the program accordingly." In its current incarnation, students work full-time in laboratories for eight consecutive weeks during the summer and then for at least three hours per day during the school year. They receive stipends for their summer internship and high school course credits for their work during the school year.

"It's hugely transformative," said Janelle Cortner, Ph.D., Scientific Program Administrator in CCR's Office of the Director. "Maybe the students have had a physics or biology lab in high school, but these are usually cookbook assignments for which the answers are known. Through the Werner H. Kirsten program, they get right into real laboratory research. By the end of the summer, there is a transformation that happens: the kids become scientists, not just students."

"My laboratory has always found them a critical asset," said Young. "Many help with screening of mice, for example. The rare student can lead his or her own project; however, you do need someone in the laboratory committed to interacting with them on a daily basis."

Students initially came from high schools within close proximity to NCI at Frederick, but the program has expanded to include students from neighboring counties and those who are homeschooled.

With a 15-hour-per-week time commitment during a student's senior year in high school, the program is not for everyone. "Through our outreach and during the interview process, we stress that this is very intense and requires a serious commitment. This is not something you do just to add it to your college application," said Cortner. "On the other hand, the best students are balanced students with good time management skills."

"I had a student who was involved with so many things—president of his senior class, captain of the football team, quarterback, that meeting his responsibilities to the program was difficult. Sometimes, he didn't show up on time and we had to sit down with him and talk about our expectations for him to succeed as an intern. A few years later, when his sister went into the program, he came up to me at the reception and told me how meaningful the program was to him. It was very gratifying to hear this, and, although he did not pursue a career in biomedical research, he was very successful in starting his own IT company," said Young.

While the program administrators have not been able to track all the students who have come through the program, at least 118 students continued on to pursue scientific or medical careers.

Amy Franciscovich, M.D., a Pediatrics Resident at The Johns Hopkins University, also interned with Young between 2001 and 2002. Franciscovich went on to complete a bachelor's degree in chemistry at Emory University, before entering Harvard Medical School. "Working at NCI was the launching point for many of my future adventures in science, giving me not only a foundation in technical skills and an understanding of laboratory equipment and methodologies, but also providing a forum for nurturing my intellectual curiosities, encouraging further inquiries and brainstorming, and teaching me how to deeply think about a specific problem while also being able to see from the 30,000-foot view," said Franciscovich. "This experience taught me that the most meaningful work comes when working with a team that values mentorship."

Applicants are matched to mentors according to mutual interest. Mentors have access to all applications and can choose those students whom they wish to interview. After the interviews, mentors and students rank their best fit, and an algorithm makes as many matches as possible.

"The only limits we have are recruiting enough mentors," said Marsha Nelson-Duncan, Education Outreach Specialist for the Office of Scientific Operations at NCI at

(Photo: R. Baer)

Howard Young, Ph.D., and Werner H. Kirsten trainee discussing lab results.

Frederick. "The mentors have made the program successful; it's part of the NCI mission to train the next generation of scientists, and the mentors take that very seriously."

Building Bridges

Dan Edelman, Ph.D., manager of the Clinical Molecular Profiling Core in CCR's Genetics Branch, has always included summer students in his laboratory, but did not anticipate starting a training program of his own. In 2009, a female student contacted Edelman through his parents to request an internship. "She was an Orthodox Jew, as am I, and during the summer of her internship, we spoke for many hours about whether she could pursue a career in science when there seemed to be too many lifestyle barriers and intellectual contradictions. There were issues that could be dealt with, but at the time, I had no structured way to address them."

Three years later, Edelman received a phone call from a science teacher at Bnos Yisroel High School, a Jewish, Torah-guided girls' school in Baltimore, Md. She was looking for opportunities for her students to see some real-world applications of what they were learning from the single science course taken each year in grades 9–11. In the summer of 2013, six female students came to Bethesda for an introduction to biomedical science. During rotations through dozens of labs and clinics, they were charged with reading scientific articles and presenting posters based on their literature review. Edelman met with them weekly to sort through their new experiences and learn how to navigate in this new environment. Of this original cohort, five returned in 2014 for an 8-week paid summer internship; three of the five returned again in 2015.

"I thought I was bringing these girls here for the science, but it was so much more. In the last three years, the girls have developed an incredible sense of confidence; they see they are able to retain their level of religiosity and yet step into the world of science, and, in this case, the world of NIH," said Edelman.

The program has continued to bring in additional interns; so far 24 students have participated. Additionally, postdoctoral and postbaccalaureate fellows serve as mentors for the first-year interns, and to ensure the best fit, they are screened through an application process. "In their own careers, they've already seen the importance of mentorship, either because they had it or didn't. Someone has to step up to the plate and help the next generation," said Edelman.

A Foot in the Door

Expanding the diversity of talent in the biomedical workforce is also the goal of the CCR Cancer Research Intern (CRI) program, which is part of the NIH Summer Internship Program. These internships are aimed at students—undergraduate and graduate—whose lack of opportunity might make them less competitive for the NIH summer internships, despite their talent and drive. The program provides one year of support only to students from groups that are underrepresented in the biomedical workforce or those from disadvantaged backgrounds. The program helps the interns network with CCR scientists to find future opportunities. Since 2004, the program has placed 272 students in CCR laboratories.

"The internship is particularly important for those students continuing toward a Ph.D. or other advanced track after an undergraduate degree because research experience is really important for a lot of graduate school selection committees," said Jonathan Wiest, Ph.D., Director of CCR's Office of Training and Education.

"The intent is to bring in individuals from a wide range of backgrounds to increase the diversity of the summer intern program and, ultimately, the biomedical workforce. Diversity brings new thoughts, ideas, and creativity to research teams. Diversity is also key to addressing socioeconomic health disparities. We still have a lot of work to do to have a diverse workforce that mirrors the population of this country."

To learn more about training opportunities at CCR, please visit the Office of Training and Education website at https://ccr.cancer.gov/training.

Always on the Move

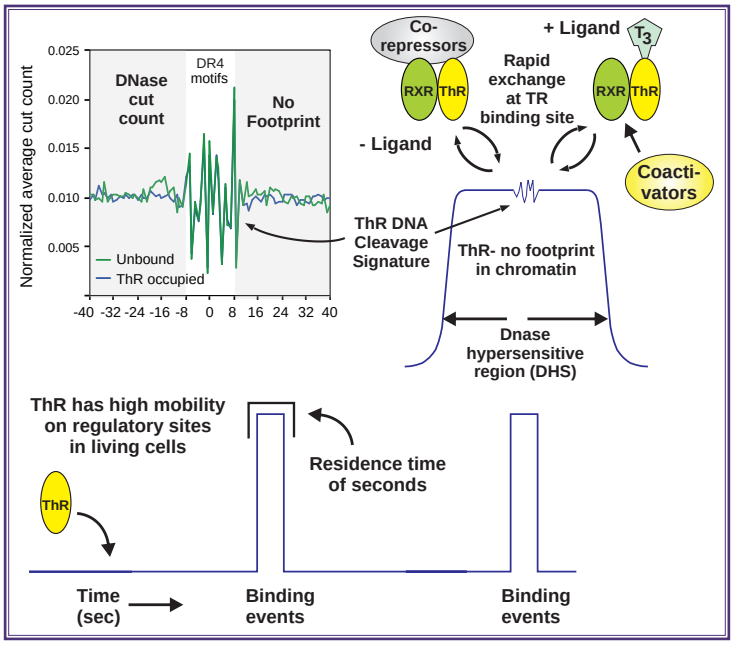
A static model of thyroid hormone receptor function is revised.

Thyroid hormone (TH) is a primary endocrine regulator of human metabolism and homeostasis. Acting through three forms of its receptor (ThR), TH regulates target gene expression in nearly every cell in the body, modulating many fundamental processes. According to the decade's-old "bimodal switch model," ThRs bind stably to chromatin at cognate recognition elements and serve as a scaffold for supercomplexes of cofactors, which activate or repress transcription. In the absence of TH, these scaffolds attract repressive cofactors; upon activation by TH, repressive factors are displaced, new activating cofactors are recruited, and target genes are induced.

Researchers led by Sheue-yann Cheng, Ph.D., Senior Investigator in CCR's Laboratory of Molecular Biology, and Gordon Hager, Ph.D., Chief of CCR's Laboratory of Receptor Biology and Gene Expression, with assistance from Paul Meltzer, M.D., Ph.D., Chief of CCR's Genetics Branch, recently challenged this view of ThR action in the mouse liver. Their findings were published in *Nature Communications*.

Combining genome-wide ChIP-seq analysis for receptor binding with DNase-seq data to monitor open and closed chromatin states, the researchers observed many *de novo* genome-binding events for the receptor. That is, rather than existing as a stable, chromatin-bound repressive factor, the receptor often moved actively to thyroid response elements (TREs) in a hormone-dependent fashion. Furthermore, the receptor often created localized open chromatin structures at the binding sites.

The researchers also monitored the stability of bound ThR. A bound factor should protect its binding site within hypersensitive regions of the DNA from degradation by DNase, resulting in a predictable footprint. None of the ThR-binding sites, either activating or repressing, showed any evidence of a corresponding footprint. ThR-binding sites were universally marked by specific cleavage signatures, which correspond precisely to the ThR DNA–binding motifs. These signatures represent non-random cleavages due to primary DNA structure.



(Figure: SY. Cheng and G. Hager, CCR)

Revised model of ThR function stresses dynamic interchange of factors on DNA.

The combined results support an altered view of ThR function, in which, the receptor exchanges rapidly and continuously with response elements in chromatin. In the absence of a ligand, the receptor recruits corepressors to binding elements, but these complexes are not statically bound to chromatin. Upon activation by the hormone, the receptor recruits coactivators, thus inducing target genes, but the receptor continues to exchange rapidly with binding elements. For steroid receptors, this mode of action has recently been termed dynamic-assisted loading.

"The nuclear receptors as a class appear to behave as highly mobile factors with the ability to initiate the chromatin transitions necessary for cofactor recruitment and enhancer action," said Cheng. "The genomic action of the thyroid hormone now appears more in alignment with well-developed models for steroid receptor action and gives us a clearer understanding of the molecular mechanisms through which this important hormone operates."

To learn more about Dr. Cheng's research, please visit her CCR website at http://1.usa.gov/1PWpj0y.

To learn more about Dr. Hager's research, please visit his CCR website at http://1.usa.gov/1WjAEgc.

Recent CCR Awards

2015 Harrington Prize for Innovation in Medicine
American Society for Clinical Investigation and the Harrington Discovery Institute

For achievements notable for innovation, creativity, and potential for clinical application

Douglas Lowy, M.D.
Chief, Laboratory of Cellular Oncology
Acting Director, National Cancer Institute

2015 Lifetime Achievement Award
International Papillomavirus Society

For important contributions to the papillomavirus research community

John Schiller, Ph.D.
Deputy Chief, Laboratory of Cellular Oncology

2015 Special Lifetime Award
Israeli Society for Bioinformatics and Computational Biology

For seminal contributions to bioinformatics research

Ruth Nussinov, Ph.D.
Senior Investigator, Cancer and Inflammation Program

ASTRO Gold Medal Award
American Society for Therapeutic Radiology and Oncology

For outstanding contributions to the field of radiation oncology

James Mitchell, Ph.D.
Chief, Radiation Biology Branch

Wilhelm Bernhard Medal
The Wilhelm Bernhard's Workshops

For major contributions to the field of nuclear architecture

Tom Misteli, Ph.D.
Senior Deputy Director for Research
Senior Investigator, Laboratory of Gene Expression and Receptor Biology

Betty Ford Lifetime Achievement Award of Distinction
Susan G. Komen Foundation

For his leadership and groundbreaking development in personalized treatment for cancer patients

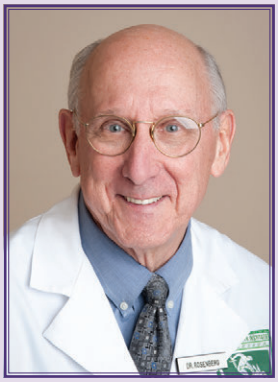
Steven Rosenberg, M.D., Ph.D.
Chief, Surgery Branch

John B. Stanbury Thyroid Pathophysiology Medal
American Thyroid Association

For outstanding research contributions to the understanding of thyroid physiology or the pathophysiology of thyroid disease

Sheue-yann Cheng, Ph.D.
Senior Investigator, Laboratory of Molecular Biology

CCR Physician-Researcher Awarded the Service to America Medal


(Photo: R. Baer)

Steven Rosenberg, M.D., Ph.D., Chief of CCR's Surgery Branch, has been named the 2015 Federal Employee of the Year by the Partnership for Public Service for his pioneering research to develop life-saving immune-based therapies for patients with advanced cancers. The award is the highest Samuel J. Heyman Service to America Medal, or Sammie.

For more than four decades, Rosenberg, has conducted research at NCI (See "Adopting Bodily Defenses to Cure Cancer," *CCR connections*, Vol. 8, No.1). He was the first to demonstrate that administering interleukin-2 could be used to effectively treat tumors in some patients with metastatic disease. He also laid the foundation for cell-based immunotherapies, specifically the development of adoptive cell transfer (ACT), which uses the body's own immune system to attack cancer cells. Rosenberg was the first to demonstrate that genetically modified T cells could mediate cancer regression in patients with melanoma, sarcomas, and lymphomas.

This medal is one of eight Sammies awarded annually by the Partnership for Public Service, a nonprofit, nonpartisan organization that works to revitalize the federal government. This year's recipients were selected from a group of over 500 nominees drawn from almost every major government agency. The Sammies have earned a reputation as one of the most prestigious awards dedicated to honoring America's civil servants and have come to be known as the "Oscars of government service."

Staff News at CCR

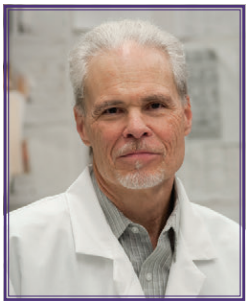
Announcements

(Photo: B. Branson)

Lee J. Helman, M.D.

Lee J. Helman has been named CCR Acting Director. He received his M.D. from the University of Maryland School of Medicine and completed his internship and residency in internal medicine at Barnes Hospital Washington University, where he served as Chief Resident. Helman began his fellowship training at NCI in 1983. He received his postdoctoral training in the Pediatric Branch and became Head of the Molecular Oncology Section, Pediatric Oncology Branch, in 1993. He served as Chief of the Pediatric Oncology Branch from 1997 to 2007 and, in 2007, became CCR's Scientific Director for Clinical Research, a position he currently holds. Helman's laboratory focuses on three major themes related to the biology and treatment of pediatric sarcomas, specifically rhabdomyosarcoma, Ewing's sarcoma, osteosarcoma, and pediatric GIST tumors: (1) determining the pathophysiologic consequences of IGF signaling; (2) identifying the molecular/biochemical determinants of the biology of these sarcomas; and (3) applying preclinical laboratory findings to the development of novel clinical studies for these sarcomas.

(Photo: R. Baer)



Glenn Merlino, Ph.D.

Glenn Merlino has been appointed CCR Acting Scientific Director for Basic Research. Merlino received his Ph.D. from the University of Michigan and then joined NCI as a Postdoctoral Fellow under Ira Pastan, M.D. He became Chief of CCR's Laboratory of Cell Regulation and Carcinogenesis in 2004 and Co-Chief of CCR's Laboratory of Cancer Biology and Genetics in 2006. Merlino has made notable contributions in the areas of receptor tyrosine kinase signaling, oncogenic transformation, transcriptional regulation, cell cycle regulation, multiple drug resistance, and genomic instability. Currently, using genetically engineered mouse models of human cancer, Merlino is seeking to elucidate the complex molecular programs governing melanoma genesis and progression. He is developing improved preclinical melanoma models to study inherent and acquired resistance to targeted and immune-based therapeutics.

(Photo: R. Baer)

Tom Misteli, Ph.D.

Tom Misteli has been named CCR Senior Deputy Director for Research. He is a Senior Investigator in the Laboratory of Receptor Biology and Gene Expression, where he leads the Cell Biology of Genomes Group. He obtained his Ph.D. from the University of London, U.K., and was a Postdoctoral Fellow at the Cold Spring Harbor Laboratory in New York. He is internationally recognized for pioneering the field of genome cell biology. His laboratory's focus is to uncover fundamental principles of spatial genome organization and to apply this knowledge to the development of novel diagnostic and therapeutic strategies for cancer and aging.

(Photo: B. Branson)

Beverly Mock, Ph.D.

Beverly Mock has been named a CCR Deputy Director. She is also Deputy Chief of the Laboratory of Cancer Biology and Genetics, and she oversees the CCR Office of Scientific Programs. Mock received her Ph.D. from the University of Maryland, and then joined CCR's Laboratory of Genetics as a Postdoctoral Fellow. She became a Principal Investigator and later Chief of the Laboratory of Genetics, which was incorporated into the Laboratory of Cancer Biology and Genetics. Mock is an internationally recognized leader in the study of complex genetic traits associated with cancer initiation and progression. Her laboratory investigates genetic susceptibility to mouse plasma cell tumors as a model system for analyzing complex genetic traits associated with cancer.

(Photo: M. Welch)

Eric Freed, Ph.D.

Eric Freed has been appointed Director of CCR's HIV Dynamics and Replication Program (HIV DRP). He obtained his Ph.D. from the University of Wisconsin-Madison, where he also conducted postdoctoral work. When Freed first came to NIH, he joined the National Institute of Allergy and Infectious Diseases. Freed joined CCR's HIV DRP in 2003 and was named Deputy Director in 2014. His research focuses on combining virology, molecular biology, and cell biology approaches to study the late stages of the virus replication pathway, specifically Gag assembly, membrane targeting, envelope glycoprotein incorporation, virus budding, and maturation. His team has also been involved in the development of HIV-1 maturation inhibitors.

New Tenure-Track Scientists

(Photo: B. Branson)

Christian Hinrichs, M.D.

Christian Hinrichs joins CCR's Experimental Transplantation and Immunology Branch as a Tenure-Track Investigator. He is also a Lasker Clinical Research Scholar. He received a combined B.A./M.D. degree from the University of Missouri-Kansas City (UMKC). He completed a residency in general surgery at UMKC, followed by a fellowship in surgical oncology at Roswell Park Cancer Institute in New York. Hinrichs then joined CCR's Surgery Branch as a Surgical Oncology Fellow. Subsequently, he completed an internal medicine residency at George Washington University and a medical oncology fellowship with CCR's Medical Oncology Branch. After completing this training, Hinrichs became an Assistant Clinical Investigator in CCR's Surgery Branch. He conducts translational research and clinical trials to develop T-cell therapies for cancers caused by human papillomaviruses.

(Photo: B. Branson)

DeeDee Smart, M.D., Ph.D.

DeeDee Smart is now a Tenure-Track Investigator in CCR's Radiation Oncology Branch. She completed her medical and graduate education at the Medical College of Georgia, followed by a Cancer Research Training Award postdoctoral fellowship in CCR's Radiation Oncology Branch. She then completed an internship in Internal Medicine at Thomas Jefferson University Hospital in Pennsylvania, followed by a radiation oncology residency at the National Capital Consortium Hospitals/NCI. In 2009, she became an Assistant Clinical Investigator in CCR's Radiation Oncology Branch. Her research focuses on the role of CNS sirtuins in mechanisms of DNA damage response in the brain and neurodegeneration resulting from radiation treatment of primary brain tumors and cerebral metastases.

(Photo: James Derek Dwyer, BIDMC)

Adam Sowalsky, Ph.D.

Adam Sowalsky joins CCR's Laboratory of Genitourinary Cancer Pathogenesis. He received his Ph.D. from Tufts University's Sackler School of Graduate Biomedical Sciences. He conducted postdoctoral training at Tufts University School of Medicine as well as at Harvard Medical School/Beth Israel Deaconess Medical Center. During his fellowships, Sowalsky was also a Senior Lecturer of Biochemistry and Genetics at Northeastern University. Following his fellowship, he was appointed as an Instructor in Medicine at the Harvard Medical School and a Staff Scientist at Beth Israel Deaconess Medical Center. The central theme of Sowalsky's research is understanding the biology of the molecular events associated with prostate cancer development, progression, and resistance to therapy. In particular, his research explores changes in gene expression that occur with each stage of prostate cancer and the genomic mutations that accompany them. In addition, Sowalsky is developing precision assays for the noninvasive detection of aggressive or recurrent disease.

Newly Tenured CCR Scientists

Tim Greten, M.D.
Thoracic and Gastrointestinal Oncology Branch

Jung-Hyun Park, Ph.D.
Experimental Immunology Branch

Roberto Weigert, Ph.D.
Laboratory of Cellular and Molecular Biology

In Conversation:

Research Fellow Joanna Sztuba-Solinska, Ph.D.

CCR: What drew you to the study of viral genomes?

Joanna: The influence of emerging pathogens like severe acute respiratory syndrome (SARS) and Ebola on global health has interested me since I was a student in Poland. At a molecular level, I am fascinated by viral resiliency and plasticity. When I came to the U.S. almost 10 years ago as a graduate student, I studied the mechanisms of RNA recombination that occur in plant RNA viruses to understand how they evolve and adjust to an ever-changing environment.

(Photo: R. Frederickson)

Joanna Sztuba-Solinska, Ph.D.

CCR: How did you come to work with Stuart LeGrice, Ph.D., Senior Investigator in CCR's Basic Research Laboratory?

Joanna: I came to the NIH in 2011 to work with Stuart because he was moving into a new field of probing RNA structural motifs in viruses. I knew that there was more to RNA than just sequence; structure had to fulfill different roles in the viral lifecycle. My very first publication in Stuart's laboratory was on Dengue virus RNA. The full genome is 11kb long, making it difficult to work with. So we use a "minigenome," which contains all essential RNA motifs that support the viral life cycle. Using a variety of biochemical probing and mutagenesis techniques, I found evidence of a tertiary RNA–RNA interaction called an H-type pseudoknot that can form transiently, like a switch that may allow the virus to use the same RNA as a template for replication or a template for translation.

CCR: How can such information be used to combat viral infections?

Joanna: The global view is to characterize structures of RNA and connect them to functions, but to also target RNA motifs with small molecules. I believe this could be a very effective way of developing new and hopefully very potent therapeutics against viruses. In fact, working with Jay Schneekloth's group in CCR's Chemical Biology Laboratory, we have been developing a method to look for RNA-specific small-molecule binders with the use of small molecule microarrays. We have already successfully applied this unique strategy to target the HIV transactivation response (TAR) hairpin.

CCR: Are collaborations important in your research?

Joanna: Definitely. I have several projects—two to three main ones at any time and then some side projects, all highly collaborative. We have a lot of meetings with people of similar interest; that's how the Dengue project began, which was a collaboration with researchers at Georgetown University Medical Center. Currently, I have collaborative projects on the Dengue virus, Ebola virus, and on the Kaposi sarcoma herpesvirus, which is a cancer-causing virus.

CCR: With so much work, how do you achieve a life balance?

Joanna: I am the mother of a 20-month-old child. My husband is a huge help—without family support, it wouldn't be possible. My daughter keeps me grounded and gives me joy after work.

CCR: What advice would you give to other NIH fellows?

Joanna: Interact with a lot of people, and discuss your ideas with your peers, principal investigators, your mentor, and your friends. Also, take some time off and be involved in different courses that NCI or NIH offers. I recently participated in a grant-writing workshop, which I believe will be very useful for me as I think about applying for academic positions. I also took a workshop on scientific management training and participated in a videocast course on translational research in clinical oncology. Don't stay closed in your lab; take advantage of opportunities.

Through the High-Tech Looking Glass

Science begins with observation; scientists have made telescopes to examine things farther away than the eye can see and microscopes to examine things invisible to human vision. Since Robert Hooke in the 17th century used the first microscope to document the existence of living cells, advances in cell biology have been tied to ever more innovative tools for visualizing and analyzing the microscopic world. CCR scientists continue to creatively expand the boundaries of observation to answer longstanding and diverse questions about the inner workings of cells.

Illuminating Discovery

When most people hear the word genome, they think about long sequences of nucleic acids strung together in a code that is read by each cell in the body. Some sequences predict eye color and some predispose to disease. But, long before its composition was known, the genome was observed as a physical structure occupying three-dimensional space inside the nucleus of every cell. Whether and how the spatial arrangement of the genome influences its function was a matter of speculation. Now we know that the positions of individual chromosomes and regions of the genome in the nuclear space are not random and their position is directly linked to their function.

(Photo: R. Baer)

Gianluca Pegoraro, Ph.D., Sigal Shachar, Ph.D., and Tom Misteli, Ph.D., in the lab

Since his first year as a Postdoctoral Fellow at the Cold Spring Harbor Laboratory, Tom Misteli, Ph.D., now a Senior Investigator in CCR's Laboratory of Receptor Biology and Gene Expression, has wanted to know what cellular factors determine where a gene is positioned in the cell nucleus. It is one of those questions in science that is fundamentally important, but the technology was simply not available to answer it—until recently. A few years ago, Misteli was instrumental in setting up the CCR High-Throughput Imaging Facility (HiTIF). The HiTIF combines state-of-the-art microscopy with the ability to automate sample handling and imaging using plates containing wells to simultaneously carry out 384 individual experiments.

"Imaging is traditionally fairly descriptive and based on a candidate approach," said Misteli. "You might examine your favorite protein in a particular biological situation and then perturb that system with a drug or genetic manipulation. I was interested in whether you could use imaging as an unbiased discovery tool.

"The concept is simple—you knock out every single gene in the genome and use an assay to figure out which one of the 20,000 genes

affects your favorite process. The ability to image a very large number of samples makes this now possible," Misteli explained.

While the concept might be simple, the execution requires the right equipment: microscopes not generally available at research institutions, robotic pipette systems for sample handling, and, equally critical, a bioinformatics group to automate the analysis of terabytes of data. "Getting the images is easy," said Misteli. "But to teach a computer to find the structures that you are interested in—and find them accurately—that is the challenge."

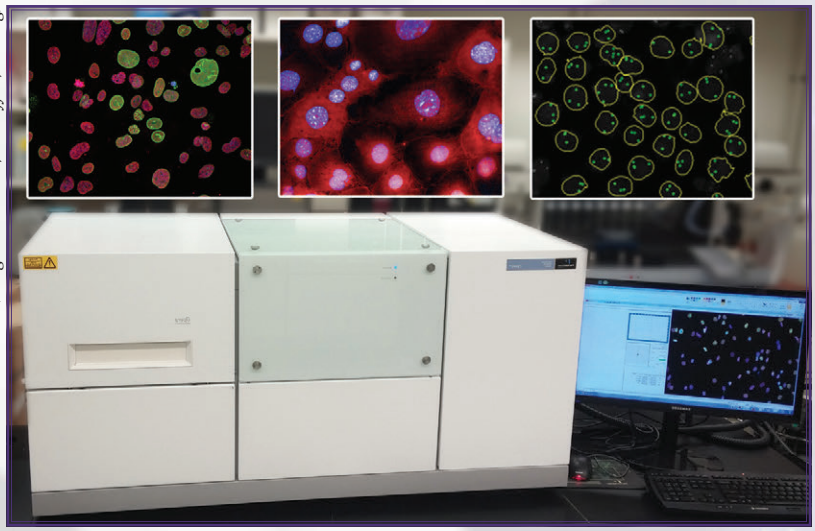
In the August 2015 issue of *Cell*, Misteli, his Postdoctoral Fellow Sigal Shachar, Ph.D., and Gianluca Pegoraro, Ph.D., Head of HiTIF, published the results of their first screen for factors controlling genome organization. Using fluorescence *in situ* hybridization (FISH) to monitor the proper positioning of a representative set of functionally diverse genomic loci, the researchers used RNA silencing to identify 50 cellular factors required for proper positioning.

"The total imaging time for that experiment was about 27 days, and we analyzed 3.5 million data points," said Misteli.

This approach, which Misteli calls Deep Imaging, is not only powerful for large-scale screening efforts, but is also equally powerful for examining rare events. "You can either take your 384-well plate and do a different experiment in every well by adding a different compound, or you can do the same experiment 384 times and look for very rare events in your very large dataset," said Misteli.

Using Deep Imaging, Misteli and his Postdoctoral Fellow, Vassilis Roukos, Ph.D., reported in *Science* in 2013 the visualization of the formation of a chromosome translocation in living cells for the first time. Chromosomal translocations, in which a segment of one chromosome is inappropriately joined to another during replication, are a hallmark of cancer cells. They are also exceedingly rare.

High-throughput imaging will also form a cornerstone of a large consortium of investigators throughout the U.S. and Europe, including Misteli, which was recently funded as part of the 4D Nucleome program, a new NIH Common Fund initiative to map the genome space and time as an extension of the Human Genome Project.



(Image: S. Shachar, P. Scaffidi, L. Fang)

High-throughput microscopes enable interrogation of a wide spectrum of intricate cellular features.

Illuminating Detail

"Centrosomes are mysterious," said Jadranka Loncarek, Ph.D., Tenure-Track Investigator in CCR's Laboratory of Protein Dynamics and Signaling. "Once you start studying them, you just fall in love."

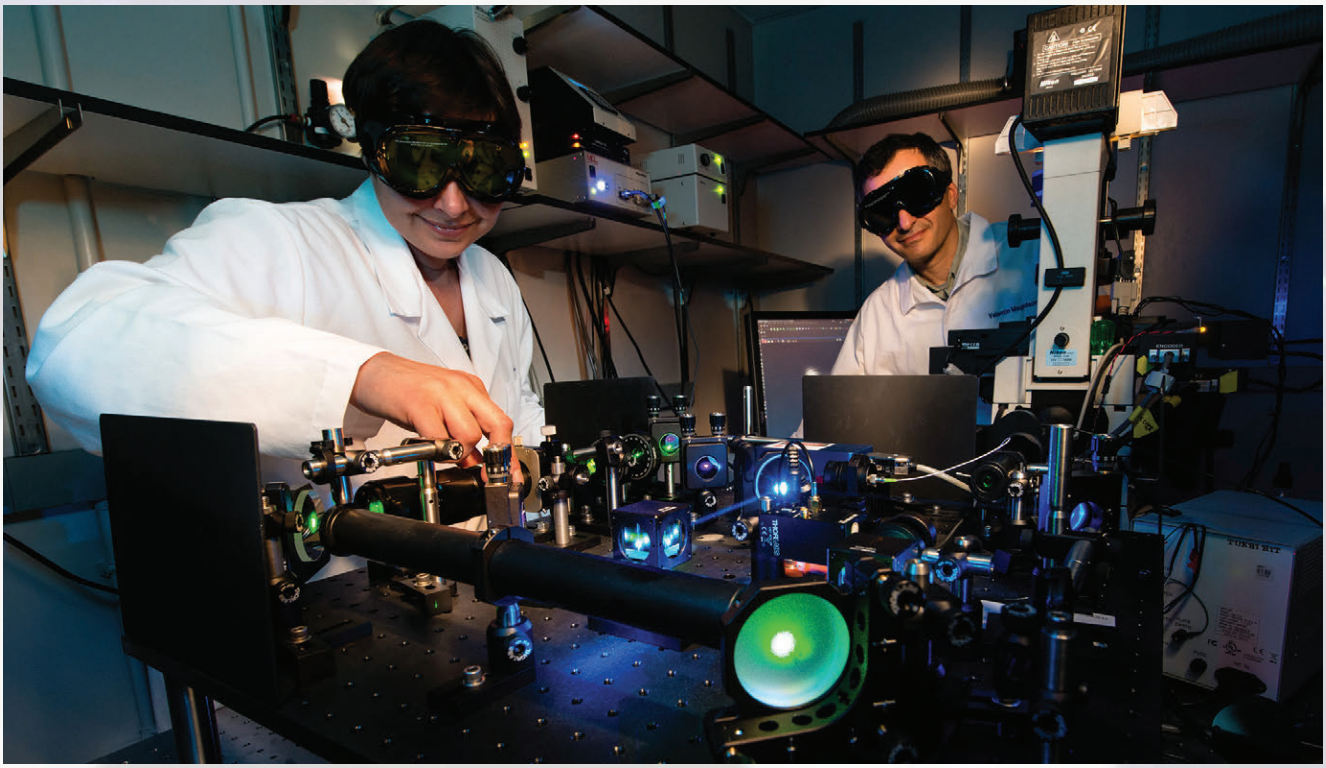
The centrosome is a complex organelle that regulates the cell division cycle. It comprises a stable core structure of a single or duplicated centriole at the center of a highly structured matrix of pericentriolar material. The centrosome is also a dynamic organelle that changes in volume during the cell cycle, and is associated with about 70–80 reliably identified core proteins and many more transiently associated ones.

"The centrosome doesn't have a clear boundary," said Loncarek. "It's an open platform for protein interactions that changes constantly during the cell cycle."

Centrosomes play critical roles in cell division; however, their own duplication processes are unusual and poorly understood. Normally, centrioles only duplicate once during the cell cycle, forming a mother– daughter pair that remains in close, orthogonal proximity. However, under certain conditions, centrioles can move away from one another and reduplicate.

But understanding centrosomes duplication is more than just a matter of academic curiosity: it turns out that most tumors have aberrations, either in the structure or number, of these organelles. Since the degree of centrosome aberrations (which in turn rests on the regulation of centriole duplication) correlates with tumor aggressiveness, the mechanisms that allow for incorrect regulation of centrosome formation are of broad interest.

Loncarek and her colleagues wanted to know what was happening at an ultrastructural level to relieve the duplication block.



(Photo: R. Frederickson)

Jadranka Loncarek, Ph.D., and Valentin Magidson, Ph.D., preparing for laser microsurgery.

Centrioles are electron-dense structures of approximately 0.5 micrometers in length, and they can be easily observed through transmission electron microscopy. However, in order for those observations to be meaningful, they need to be matched with observations of the cell at the light microscope level.

Loncarek uses a method called correlative light and electron microscopy (CLEM). "It is not easy," said Loncarek. "First, you study the cell under the light microscope, and then you have to find that same cell among thousands after all the preparation necessary for electron microscopy. It's a very error-prone technique. You need to develop expertise in both light and electron microscopy to bridge the gap."

After almost two years of troubleshooting, Loncarek and her Postdoctoral Fellow, Dong Kong, Ph.D., have overcome the technical hurdles to making CLEM a routine and reliable part of their research strategy. Using this technique, Loncarek's team found that beyond a critical distance of only 80 nanometers between mother and daughter centrioles, the mother centriole can undergo reduplication. Moreover, contrary to widely held beliefs, they showed that the orthogonal positioning was not important for blocking duplication. Their findings were published in *Nature Communications* in August 2015.

"We would really like to understand this distancing process between mother and daughter, such that the latter slowly walks away until the mother doesn't feel it any more as an inhibitory element for its reduplication. We are designing experiments to study the molecular mechanism behind this process," said Loncarek.

Loncarek and her team are now working with super-resolution microscopy to study the organization of proteins around the centriole. Stochastic optical reconstruction microscopy (STORM) relies on fluorescent probes that switch rapidly between light and dark states. A single snapshot shows only a small subset of fluorescent spots, whose centers pinpoint position with high accuracy. A final composite image, therefore, has higher resolution than an image in which all loci were simultaneously labeled.

"STORM allows us to use mathematical algorithms to reconstruct images with up to a 10-nanometer resolution. However, the sample preparation is complicated, and only certain dyes can be used," said Loncarek. "A lot of people are working to develop better probes we can use in live cells and then in super-resolution microscopy. The current great challenge is to find a probe that maintains sufficient fluorescence, even after processing, for electron microscopy for direct comparison of protein localization with ultrastructure."

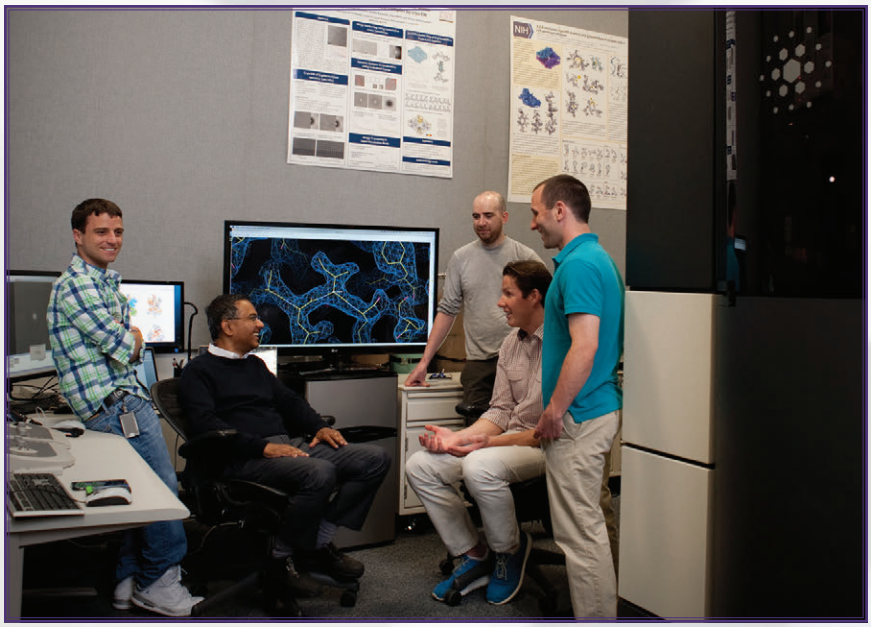
Illuminating Molecules

"Why shouldn't it be possible to look at the 3D structure of a protein like you look at a cell through a microscope?" asked Sriram Subramaniam, Ph.D., Senior Investigator in CCR's Laboratory of Cell Biology. Subramaniam has spent more than a decade bridging the technological gaps that stand in the way of solving interesting biological problems, using high-resolution electron microscopy at the interface between structural biology and cell biology. (See Box: Center for Molecular Microscopy).

Most recently, his laboratory has focused on cryo-electron microscopy as a means to access the structure of proteins at atomic resolution, while suspended in solution. Many biologically important proteins have not been amenable to classical techniques of structural biology because of their size or their conformational heterogeneity.

Given recent advances in electron detectors, it is now possible to take images of individual molecules at higher definition than ever before using a transmission electron microscope. Because electron beams are damaging over time, each particle is only viewed very briefly, leaving a grainy image. However, by averaging images of tens of thousands of particles, it is possible to computationally create a three-dimensional picture of the particle. It is now even possible to categorize these images, deriving separate three-dimensional structures for the particle in several different conformations at the same time.

Subramaniam and his colleagues have used this technique to study mechanisms of ion-channel gating and enzymatic regulation. Earlier this year, they described, in *Science*, the structure of the bacterial enzyme β-galactosidase in complex with an inhibitor at a resolution of close to 2 Å. In addition, the Subramaniam lab has a new manuscript in press in *Cell*, which describes new mechanistic insights into a 200kDa ion channel, one of the smallest membrane proteins yet studied by cryo-electron microscopy.

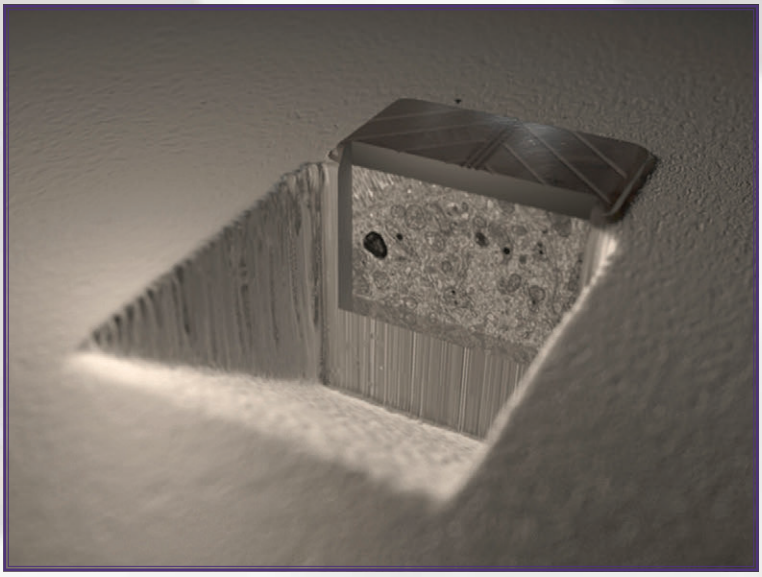


(Photo: R. Baer)

Sriram Subramaniam and members of his team discussing the Krios microscope with FEI Company representatives. Left to right: Alan Merk, Sriram Subramaniam, Ph.D., Kieran Moynihan, Gijs Janssen, and Joseph Darling, Ph.D.

Subramaniam has now turned his attention to cancer, studying complexes of small molecules with cancer targets, with the goal of influencing drug design at an early phase.

"There is a lot of information we can obtain about the interaction of drugs with the molecules they target," said Subramaniam. "So far,



(Image: D. Bliss, NIH, and S. Subramaniam, CCR)

A resin-embedded cell is excavated with an ion beam, then imaged with a scanning electron beam. Its surface is abraded with the ion beam and again imaged in an iterative process. Layer by layer, images are collected and then aligned to form a 3D volume. Here, the exposed face of the specimen is shown at the back of a trench created by the ion beam. A protective carbon pad overlays the embedded cell where future imaging will take place.

> "It is now possible to take high-definition images of individual particles—that have been trapped in a frozen solution—with a transmission electron microscope."

structural biology has been useful for snapshots, but if you could get the same information in a more physiological context and bound to its molecular partners, that information would be much more incisive in making choices about which drugs might work."

Illuminating the Future

"We are fortunate to work in an institute that supports not just the application, but also the development of new technologies," said Misteli. "Given the large investments involved, very few academic institutions have developed the imaging resources we now use routinely."

Loncarek noted that the investment is not only in equipment, but also in personnel time. "We combine biochemistry with high-end imaging. We spend a lot of time training to become experts in both."

Ultimately, the investment pays off many times over, as scientific progress is intimately tied to technology development. For example, demand for access to the Center for Molecular Microscopy, which builds on technologies developed in Subramaniam's laboratory, already exceeds capacity. "The center is a way for other CCR investigators to benefit from the investment made in my program," said Subramaniam.

To learn more about Dr. Misteli's research, please visit his CCR website at http://1.usa.gov/1NdIq0T.

To learn more about Dr. Loncarek's research, please visit her CCR website at http://1.usa.gov/1Nrjjw3.

To learn more about Dr. Subramaniam's research, please visit his lab's website at http://electron.nci.nih.gov.

Center for Molecular Microscopy

Ten years ago, Sriram Subramaniam, Ph.D., described four classes of problems that could be solved by advances in imaging:

1. Is it possible to distinguish cell architectures, for example in cancer, by quickly determining and comparing three-dimensional structures of whole cells?
2. How do we describe how receptors and signaling assemblies change in a concerted way, in response to external signals?
3. Can we determine the structures of proteins and other entities, such as HIV, that cannot be crystallized?
4. Why can't we just look at the structure of a protein in different conformations?

Over the next 10 years, his laboratory implemented new approaches, including focused ion beam scanning electron microscopy, whole-cell tomography, and cryo-electron microscopy, to address these questions.

"Two years ago, I saw an inflection point in the field where some of the techniques we were developing would go from niche applications in specialized laboratories to being more widespread. The Center for Molecular Microscopy (CMM) is an experiment to transition technology from my laboratory into a collaborative environment, which lets others within CCR experience and eventually adopt the technology," said Subramaniam.

CMM is staffed with dedicated scientists, many of whom trained with Subramaniam, who work with CCR scientists on their research questions.

"It's not like a core facility with a confocal microscope or a gene sequencer. Its goal is to enhance the research that others are doing on interesting biological problems by providing a unique technological perspective," he explained.

Proof that this approach can yield rich dividends is already evident. Two publications recently resulted from collaborations between CMM staff and research groups at the NIH: the three-dimensional structure of mitochondrial networks in muscle was published in the July 30, 2015, issue of *Nature*, and the structure of bacterial spores was published in the April 9, 2015, issue of *Nature Communications*.

To learn more about the Center for Molecular Microscopy, please visit its website at https://cmm.nci.nih.gov.

FEATURE

Viral Activity

In the last four decades, HIV has gone from being an unknown killer to the cause of a manageable chronic disease. Stephen Hughes, Ph.D., Chief of CCR's Retroviral Replication Laboratory, began his study of retroviruses before HIV was identified, but quickly made the virus the main focus of his research career. Hughes is internationally recognized for his work on two of the three essential enzymes in the HIV life cycle: reverse transcriptase (RT) and integrase (IN). His work has shed light on the emergence of drug resistance and, more recently, the nature of reservoirs of HIV that persist despite combination antiretroviral therapy. He has also used engineered host proteins that redirect HIV integration as tools for understanding eukaryotic chromatin organization.

If asked, Stephen Hughes will tell you that the retrovirus HIV is a fascinating creature, marvelous in its complexity. "It's only 10 kilobases. You could memorize the sequence of its nucleic acids; you could have it built for you. But knowing all that it does to survive is still far beyond us," said Hughes.

Hughes committed to studying retroviruses after completing his graduate training in the laboratory of Mario Capecchi, Ph.D., who later won a Nobel Prize. He viewed retroviruses as primarily a tool for understanding how genes worked in higher eukaryotes. "It seemed to me, at the time, that retroviruses were probably masquerading as genes in their integrated state," said Hughes. He arrived as a Postdoctoral Fellow to work with the future Nobel Laureates, Harold Varmus, M.D., and Michael Bishop, M.D., at the University of California, San Francisco (UCSF), at what he describes as a magical moment. "I showed up in 1976, and, when I left three years later, the fundamental questions about how the RNA was organized and proteins were made had been answered."

(Photo: R. Frederickson)

Stephen Hughes, Ph.D.

During his years in San Francisco, men in the Castro district where he and his wife lived were just beginning to die of what was then termed GRID for "gay-related immune deficiency." By the time HIV was identified as the probable cause of AIDS, Hughes had recently arrived at the Advanced Bioscience Laboratories—Basic Research Program at NCI at Frederick, under the direction of George Vande Woude, Ph.D., who nudged Hughes in the direction of HIV.

Reverse Transcriptase as a Drug Target

Hughes was interested in studying retroviral enzymes. Two key steps distinguish the retroviral life cycle: 1) the genome is RNA that is converted, in infected cells, into DNA through the actions of RT, and 2) the DNA is permanently embedded in the host genome through the actions of IN.

Working with a visiting scholar from Israel, Amnon Hizi, Ph.D., Hughes succeeded in using recombinant DNA in *Escherichia coli*

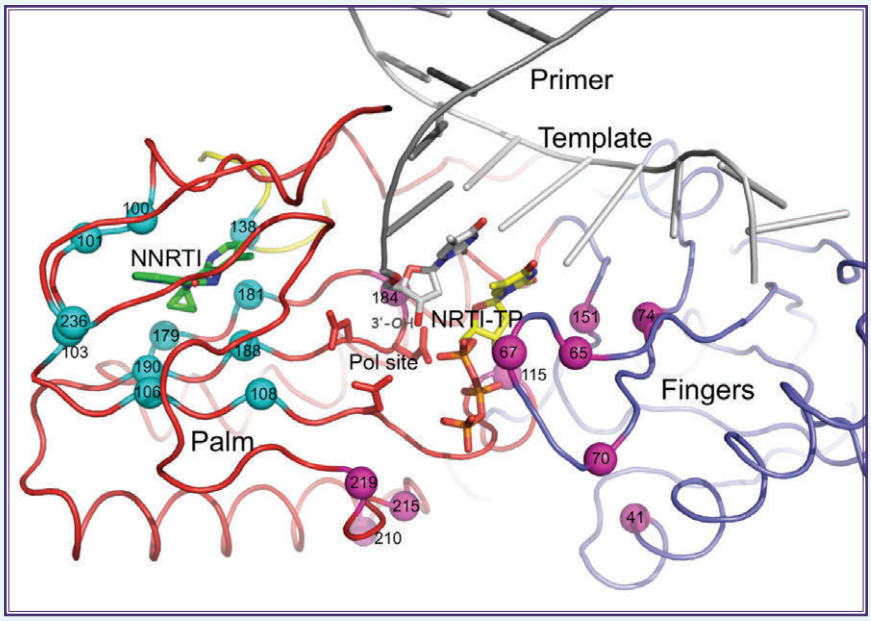
to produce RT from the murine leukemia virus (MLV) in useful quantities. "George Vande Woude came to talk with us because we were wildly excited about the amount of MLV RT we had purified," said Hughes. "George said, 'This is really good. I don't mean to throw cold water on your efforts, but you should probably do this for HIV.'"

HIV RT was more challenging to express and purify, but Hizi, Hughes, and their colleagues overcame the obstacles. As in the case of MLV, however, RT was much more tractable than the two other key HIV enzymes. "Protease was toxic to *E. coli* and integrase had unfortunate physical properties, but we had an active, soluble RT," said Hughes. Meanwhile, the nucleoside analog AZT, acting on RT, was found to be the first highly effective anti-HIV drug.

"It was obvious to retrovirologists that as soon as you began to treat HIV with drugs, you would get resistance," said Hughes. "So we thought having large quantities of purified HIV RT would give us a tool to study resistance biochemically and, with some luck, structurally."

It took some effort to persuade structural biologists to share this view. "When we began making milligram quantities of RT, I literally couldn't give it away to prominent crystallographers. They all had their own proteins, which they thought were more interesting," said Hughes.

Fortunately, he met Eddy Arnold, Ph.D., who was excited by the challenge of crystallizing RT. The Hughes and Arnold laboratories worked together for about four years, until eventually they were able to form good crystals of HIV RT that could be used for structural analysis. RT is a physically flexible protein, which resists the orderly stacking that is so important for X-ray crystallography. "We used some tricks to help stabilize the protein," said Hughes. "We made a family of monoclonal antibodies, and Eddy and his colleagues cocrystalized RT with an antibody fragment and a nucleic acid substrate to improve the structure."



(Figure: K. Das and E. Arnold, Rutgers)

The structure of HIV-1 reverse transcriptase (RT). Image shows a close up of the region around the polymerase active site where mutations can cause resistance to anti-AIDs drugs. The RT backbone is shown as a wire diagram, and the p66 fingers subdomain is shown in blue and the palm is in red. In this image, the dsDNA is shown as two wires with branches to represent the bases. The incoming dNTP is shown as a wire frame model. Positions in RT where mutations give rise to resistance to nonnucleoside inhibitors (NNRTIs) are shown in light blue, sites where mutations give rise to resistance to nucleoside analogs (NRTIs) are shown in purple.

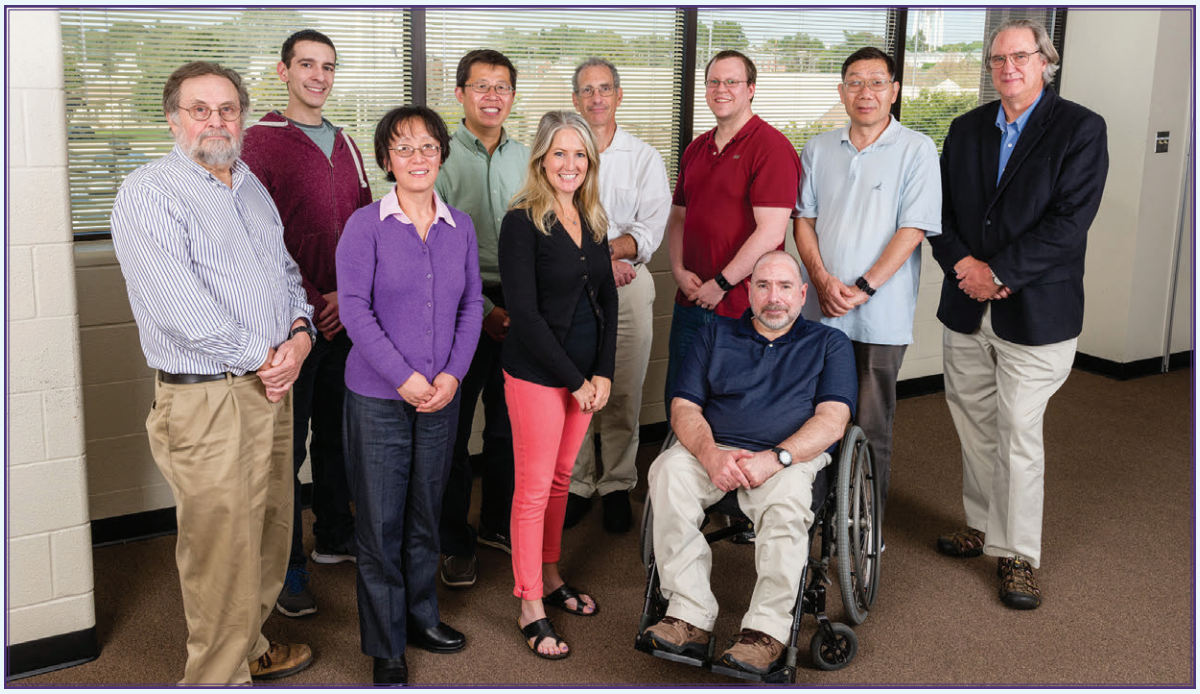
Arnold and Hughes worked together for more than 25 years on understanding the structure and function of HIV-1 RT, how drugs inhibit the enzyme, and how resistance mutations overcome the actions of different drugs. Arnold's lab has analyzed the structure of the wild-type and mutant RT proteins, and Hughes' lab has done the biochemistry and virology of the same mutants.

Some months after their collaborative efforts began, Hughes was surprised to see the tide turning, as other crystallographers began to reach out to him to obtain HIV RT for structural studies. It transpired that Marvin Cassman, Ph.D., National Institute of General Medical Sciences, started a new program to support structural work on HIV proteins, through which Hughes and Arnold were able to continue their ongoing research. "Marvin had the deep insight that understanding the structure of HIV proteins would be important. Several important protein structures came from this initiative. It was one of those instances where a single person changed how things were done in the field," said Hughes.

Integration as a Tool

"I have always had a soft spot for integration," said Hughes.

During his postdoctoral work, Hughes solved the structure of the provirus, the viral DNA that is integrated into the host genome, but when he established his own laboratory, most of the work was focused on other problems. When the work in his laboratory shifted to HIV, technical hurdles prevented him from working on the enzyme central to integration, IN. "We did play with it a couple of times, trying to do experiments in parallel with our work on RT. It was just intractable. We set IN aside for a long time," said Hughes.



(Photo: R. Frederickson)

The HIV integration site research team. Front row (left to right): John Coffin, Ph.D., Ling Su, M.S., Mary Kearney, Ph.D., and Shawn Hill, M.S. Back row (left to right): David Wells, M.S., Xiaolin Wu, Ph.D., Frank Maldarelli, M.D., Ph.D., Jonathan Spindler, B.S., Wei Shao, Ph.D., and Stephen Hughes, Ph.D. Not shown: John Mellors, M.D., Francesco Simonetti, M.D., and Andrea Ferris, M.S.

The HIV provirus integrates into host DNA by forming a poorly defined preintegration complex (PIC), which interacts with a chromatin-associated protein, lens epithelium-derived growth factor (LEDGF). LEDGF is a bipartite protein; one end has two sequences that interact with histone modifications and DNA, respectively, and the other end interacts with IN in the PIC. LEDGF preferentially directs HIV integration to the sequences of highly expressed genes.

"Eric Poeschla (then at the Mayo Clinic, now at the University of Colorado, Denver) did an experiment which just floored me," said Hughes. "He showed that if he took away the nucleic acid and histone binding component of LEDGF and replaced it with something else that would also bind chromatin, the resulting fusion protein still enabled efficient HIV integration."

Poeschla's experiment immediately suggested to Hughes that not only would the fusion protein preserve integration efficiency, but it could also direct that integration to different genomic sites depending on the specificity of the engineered chromatin-binding component. This integration could be important not only for gene therapy applications, where integration into the wrong piece of DNA can have disastrous effects, but also for chromatin mapping. In 2010, Hughes and his colleagues published, in *PNAS*, the finding that substituting two different chromatin-binding domains (CBDs)—the plant homeodomain finger from inhibitor of growth protein 2 (ING2) and the chromodomain of heterochromatin protein 1-α (HP1α)—directed HIV to different integration sites according to their known specificities.

Thus, determining the sites of HIV integration could be used as a tool to map where the fusion protein binds to chromatin. Hughes collaborated with Xiaolin Wu, Ph.D., Senior Scientist at Leidos Biomedical Research, Inc., to develop the technique, which they called HIV integration targeting (HIT-seq). In 2013, in collaboration with Robert Roeder (Rockefeller), they published a paper in *Cell*, in which they used HIT-seq to describe the effects of a common histone modification on p53-dependent transcription of active genes.

"In order to get HIT-seq to work, we had to be reasonably efficient at recovering the integration sites. It was a considerable amount of work, but we got good results using Illumina deep sequencing," said Hughes. "So we wondered if we could take this ability back to our HIV research and study where HIV integrates in patients."

"Thus, determining the sites of HIV integration could be used as a tool to map where the fusion protein binds to chromatin."

Integration and Disease

"Why can't we cure a patient with HIV?" asked Hughes. "If you can completely suppress viral replication in patients with combination antiretroviral therapy (cART) for eight to ten years, why don't all the virally infected cells die?" Many have suspected that long-lived memory T cells are a reservoir, but data from the study of HIV integration sites in patient cells, published last year in *Science*, suggests a more disturbing possibility to Hughes and his colleagues.

HIV DNA integration can occur at millions of different sites in the host DNA. Thus, if two cells have identical HIV integration sites, they were probably derived from the same originally infected cell. Hughes and his colleagues sequenced the HIV DNA integration sites in peripheral blood mononuclear cells (PBMCs) or CD4+ T cells from the blood of five patients treated with long-term cART. Of the 2,410 integration sites they identified, approximately 40 percent were found multiple times, showing that these sites came from cells that had clonally expanded after infection. In one striking example, more than 50 percent of the infected cells in a patient were from a single clone. Moreover, some of the clones of HIV-infected cells were shown to persist in patients for more than a decade.

More recently, Hughes and his colleagues have gone on to show that the virus from the expanded clone is produced at low levels in the patient and is capable of replication. "People had assumed that cells were infected and went to sleep, but suppose that's not true? Suppose there is a population of clonally expanding cells, but they do not all behave identically, and only a small fraction are actively making virus at any one time?"

Perhaps more surprising than the presence of clones was that the data from these patients also showed there was selection for cells with integration sites in specific portions of two of the genes, *MLK2* and *BACH2*, where there were, respectively, 16 and 17 independent integrations. The sites and orientations of the integrations in *MKL2* and *BACH2* suggested that these integrations altered the expression or the protein products produced by these two genes. Meanwhile, in control experiments performed by infecting cultured cells with HIV, there was no preferential integration in one orientation in either *MKL2* or *BACH2*, nor was there preferential integration in the target regions of these genes. Thus, cells with integration sites in these two genes appeared to have gained a selective growth and survival advantage.

"Most of us were reasonably convinced we would find clones of infected cells," said Hughes. "But we weren't prepared for the fact that HIV integration could drive clonal proliferation of the cells. We are quite confident that in the case of *BACH2* and *MKL2*, integration of the provirus is a major contributor. It remains to be seen what fraction of the other integration sites are driving proliferation."

Much more work is needed to establish the importance of these cells to the course of the disease. And, if one believes they are important, the questions turn to when these cells start to expand and where they persist.

Meanwhile, Hughes is also turning his attention to the implications of the integration work on cancer. In mice, MLV integration into the *BACH2* gene is known to cause tumors. In people, cancer is usually a multistep process that may not have had the time to develop in untreated HIV patients. However, in the last 10–15 years, better anti-HIV therapies have allowed patients to start to achieve relatively normal life spans. The higher rate of cancer in HIV-infected patients is usually attributed to the failure of the immune system to control herpes viruses. The question is whether all cancers will be attributable to that cause, and, if not, what role (if any) HIV integration sites might be playing.

Despite these looming questions, Hughes sees the progress that the field of HIV research has made over the last 30 years as a testament to human ingenuity and a matter of fortunate, if imperfect, timing.

"I think there is no question that HIV jumped from chimps to humans in West Africa around 100 years ago," concluded Hughes. "Imagine if that had happened 100 or 150 years earlier. We would have been intellectually and medically completely unprepared. As bad as it is now, it would have been much more severe. Conversely, I think if it had appeared 100 years from now, it would not have been a difficult problem to resolve. If you wanted to imagine a problem that was just beyond our intellectual grasp, and one that would make us work as hard as we could and reach as far as we might, with important consequences for millions of people, the rise of HIV is it."

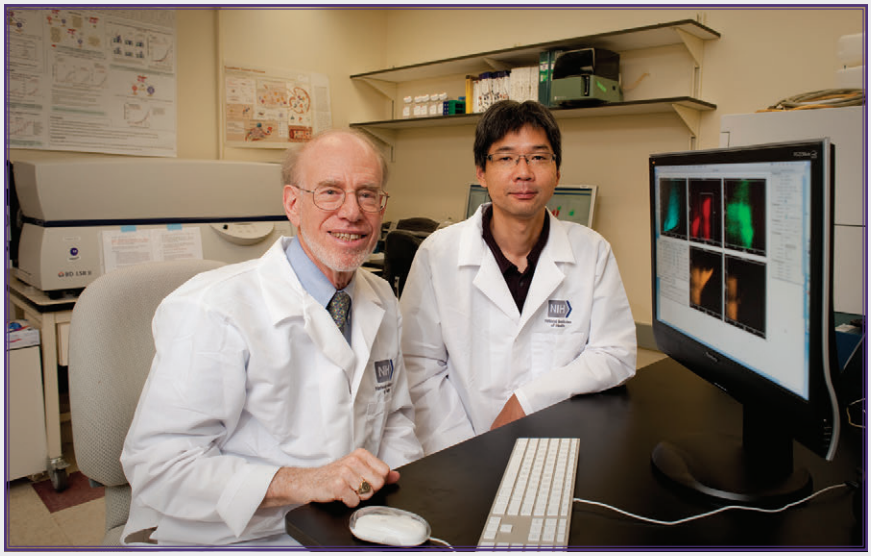
"HIV DNA integration can occur at millions of different sites in the host DNA."

To learn more about Dr. Hughes' research, please visit his CCR website at http://go.usa.gov/3JG5W.

Vaccines 2.0

In 1974, Jay A. Berzofsky, M.D., Ph.D., now Chief of CCR's Vaccine Branch, came to NIH to study protein folding. His curious mind and collaborative spirit quickly led him into the intertwined fields of immunology and vaccine development. With close to 500 publications to his name, Berzofsky has pioneered the characterization of B- and T-cell epitopes and their modification to make vaccines directed against cancer and chronic infectious diseases. He has also characterized and taken advantage of the cellular and molecular regulators of immune responses in order to enhance tumor immunity and vaccine efficacy. In the last several years, he has translated many of these strategies into promising clinical trials. From the microcosm of his laboratory, he brings the same spirit of cross-fertilizing, bench-to-bedside research to leading the Vaccine Branch as a whole.

Conventional vaccines have been extremely effective against disease agents, especially viruses, which cause acute, self-limited infection. In unvaccinated populations, diseases such as smallpox and measles are sometimes fatal, but if the immune system clears the virus, individuals usually have long-lasting immunity against future infection. Vaccine development, therefore, has classically been directed by virologists, who design attenuated agents that are safer than the original virus, but are capable of recreating the immune response.



(Photo: R. Baer)

Jay A. Berzofsky M.D., Ph.D., and Masaki Terabe, Ph.D.

In the case of both cancer and HIV, vaccine development has proven much more challenging, and, according to Berzofsky, the challenges are for similar reasons. "Both cancer and HIV cause chronic disease," said Berzofsky. "If the immune response to the virus could clear HIV, it would have done so. Therefore, the natural virus itself is not an adequate vaccine. Similarly, it may be that a lot of cancers are eliminated by the immune system, but for the ones that are not, we need better stimulation than the tumor provides. Vaccine development has shifted from the domain of the virologist to that of the immunologist."

Berzofsky's interest in vaccine development evolved from studying enzymes and protein structures. Arriving at the NIH for a postdoctoral fellowship in the laboratory of Alan Schechter, M.D., and Chris Anfinsen, Ph.D., just two years after Anfinsen won the Nobel Prize for his work on protein folding, Berzofsky got involved in a collaboration with David Sachs, M.D., then a new investigator in NCI's Immunology Branch. "I became fascinated with immune response genes," said Berzofsky.

Immune response genes are now known as major histocompatibility complex (MHC) genes, and in humans the MHC molecules are

> "...the immune system has this exquisite specificity to see differences in single amino acids in a protein."

given the name human leukocyte antigen (HLA) molecules because of the way they were first discovered. These molecules bind fragments of pathogens, i.e., epitopes, and present them on the cell surface for recognition by T-cell receptors (TCRs). In the 1980s, Berzofsky began collaborating with Charles DeLisi, Ph.D., in NCI's Mathematical Biology Branch, on algorithms for predicting which amino acid sequences would serve as epitopes by binding to MHC molecules and TCRs, and developed one of the first successful ones. To complement the modeling studies, they started doing amino acid substitution and binding studies to dissect out which residues interacted with MHC molecules and which interacted with TCRs.

"In the course of that work, we discovered that, in some cases, we could improve on epitopes and make them even more potent by getting rid of or substituting a side chain," said Berzofsky. "If you imagine the peptide is like a hotdog in a bun—between the MHC and the TCR—and you can change just the amino acids on one side of the bun that will improve binding to the MHC, then you could have more potent antigen-eliciting T-cell responses, which would also be effective against the native protein."

Immune Activation

And thus, the concept of epitope enhancement was born. Berzofsky's laboratory applied this strategy to many infectious diseases, including malaria, collaborating with Lou Miller, M.D., Section Chief in the National Institute of Allergy and Infectious Diseases' (NIAID's) Laboratory of Malaria and Vector Research. Around this time, HIV was discovered. While working with the NCI laboratory of Robert Gallo, M.D, and in collaboration with Gene Shearer, Ph.D. in NCI's Immunology Branch, Berzofsky's group obtained unpublished sequences from HIV proteins and described some of the first T-cell epitopes for the virus. Collaborating with Stephen Feinstone, M.D., at the U.S. Food and Drug Administration, Berzofsky's team published one of the first epitope-enhancement papers as a strategy to improve a vaccine against hepatitis C.

"We were working on viruses, then viruses that caused cancers, and, eventually, we started looking at cancer antigens themselves," said Berzofsky. "I had the idea that you should be able to target an antigen that is unique to cancer. Most conventional chemotherapies are poisons, but the immune system has this exquisite specificity to see differences in single amino acids in a protein. We could have a more effective and much safer therapy for cancer that wouldn't have side effects. That's what really excited me."

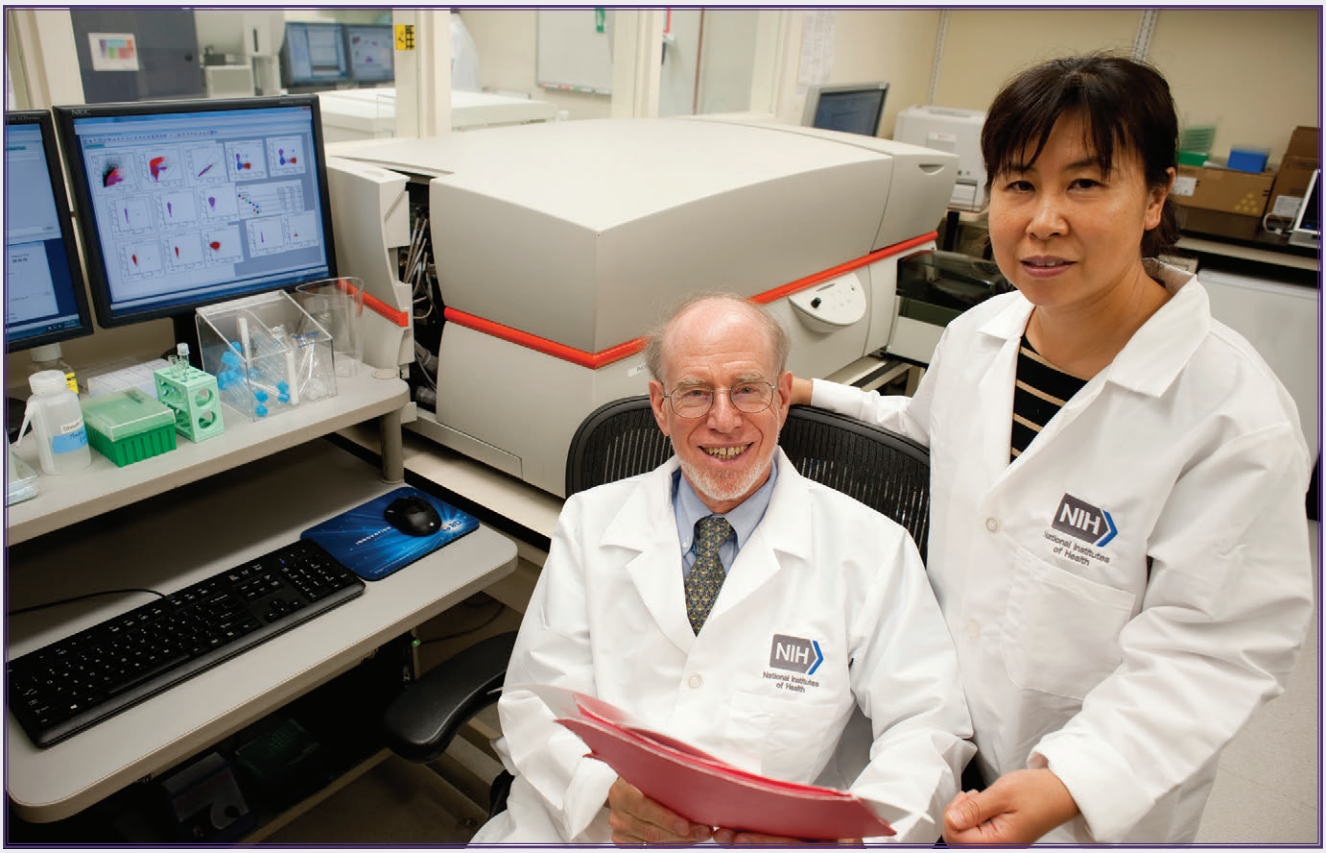
Berzofsky started looking for tumor antigens. He formed a collaboration with John Minna, M.D., then at NCI, and his fellow, David Carbone, M.D., Ph.D., to study mutations in RAS and p53, which led to a proof-of-concept human trial in which the group created an individualized peptide vaccine based on sequences from each patient's tumor biopsy. Working with John Morris, M.D., Staff Clinician in CCR's Metabolism Branch, Berzofsky's team created another vaccine that they have just translated to a clinical trial run by Lauren Wood, M.D., Head of the Vaccine Branch Clinical Trials Team, based on an adenovirus that expresses nononcogenic domains of HER2, the receptor expressed in 25–30 percent of breast cancers as well as a smaller percentage of many other cancer types. Meanwhile, they also started working with Ira Pastan, M.D., Co-Chief of CCR's Laboratory of Molecular Biology, who was mining a database of genes expressed in cancer for a different purpose, namely to find cell-surface targets for immunotoxins.

"Ira gave us sequences of the tumor antigens he discovered, and we mapped epitopes initially presented by HLA-A2 because it is the most common. We applied epitope enhancement to improve binding to the HLA-A2 molecule and enhance immunogenicity. One of these cancer antigens was TARP," said Berzofsky.

TARP (T-cell receptor gamma chain alternate reading frame protein) is expressed in the normal prostate and overexpressed in prostate and breast cancers. In 2004, the team published preclinical evidence from transgenic mice and in cells from a patient with prostate cancer, showing that their TARP epitopes could stimulate T-cell responses that killed human tumor cells.

"It took a number of years to translate those results into human clinical trials, but we have recently completed one trial and are starting another," said Berzofsky. "We are very excited about the possibilities for the TARP vaccine."

For their phase I trial, Wood enrolled 40 patients with stage D0 prostate cancer for treatment with TARP-primed autologous cells. At stage D0, the primary tumor has been removed or treated with radiation to completely destroy the tumor and the prostate. A certain



(Photo: R. Baer)

Jay A. Berzofsky M.D., Ph.D., and Yongjun Sui, Ph.D.

fraction of patients are cured, but many are not.

"After a year, we had a decreased tumor growth rate in about three-quarters of our patients. Based on these promising results, we've set up a randomized placebo-controlled phase 2 study, which is currently in progress. If we confirm what we saw in phase 1, we hope to be able to try the vaccine in more advanced prostate cancers," said Berzofsky. "It's very gratifying to see work we did at a very basic level, beginning with epitope mapping 30 years ago, now translated into human clinical trials that could benefit patients."

Immune Regulation

"The very first work I did here at NCI was to understand how the immune response against cancer is regulated, and the result we got was totally unexpected," said Masaki Terabe, Ph.D., who arrived in Berzofsky's laboratory as a postdoctoral fellow in 1999 and is now Deputy Section Chief. "Even Jay was surprised."

Working with the laboratory of Ron Germain, M.D., Ph.D., then a Principal Investigator in NIAID's Laboratory of Immunology, Berzofsky's lab had created a mouse model in which tumors would grow, start to regress spontaneously, and then recur. Not surprisingly, they found, through cell-depletion experiments, that the regression was dependent on CD8+ T cells, but they were surprised to find that the recurrence of the cancer was due to CD4+ T cells, specifically a subset defined as type II NKT cells, which recognize lipid antigens presented by a nonclassical MHC molecule.

"Masaki made the groundbreaking discovery that NKT cells could inhibit tumor immunity," said Berzofsky. "And he discovered the mechanism involved IL-13 and TGF-β. This really opened up a whole new area of immune regulation for our laboratory."

After Terabe showed that TGF-β was a critical mediator of immune suppression in this system, the team began using an antibody against TGF-β to prevent growth of tumor models, both independently and in synergy with cancer vaccines. As a result, Berzofsky has worked first with Genzyme and now with Xoma to bring anti-TGF-β into the clinic. A phase 1 clinical trial for melanoma sponsored by Genzyme gave encouraging results, but changes in corporate priorities ended that line of investigation. Now, Berzofsky has a Cooperative Research and Development Agreement (CRADA) with Xoma, under which his lab is completing preclinical mouse studies with a new set of anti-TGF-β antibodies.

"Our work with TGF-β is one part of an overall 'push–pull strategy' to improve the T-cell response to cancer. We are using defined molecular

adjuvants to push and steer the response in the right direction and the blockade of negative regulators to take the brakes off the response and thus 'pull' it forward," said Berzofsky. A key molecular adjuvant is IL-15, which Berzofsky studied in collaboration with Thomas Waldmann, M.D., now Chief of CCR's Lymphoid Malignancies Branch, who was Berzofsky's former mentor (See "IL-15 Prepares for Its Clinical Debut," *CCR connections* Vol. 5, No.2).

Meanwhile, at a more fundamental level, Terabe and Berzofsky are working on finding a good marker for type II NKT cells. "They are very rare; if you draw blood, you find only one in a thousand to ten-thousand cells," said Terabe. "We are trying to establish a method to identify them reliably. We still know very little about these cells."

Immune Compartmentalization

Even while cancer immunology has blossomed in Berzofsky's laboratory, HIV vaccine research has not languished. What began as the study of T-cell epitopes in unpublished sequences from the Gallo laboratory has become focused on ways to defeat HIV at the mucosal membrane.

"Eighty-five percent of HIV/AIDS is transmitted vaginally or rectally. HIV is a mucosal disease," said Yongjun Sui, Ph.D., Staff Scientist leading Berzofsky's HIV mucosal vaccine team. "There are systemic antibodies in the blood that could protect against flu or other infectious diseases, but the first line of defense for HIV is at the mucosal surface. Our idea is to develop the T-cell or antibody response at the point of entry."

In the 1990s, Berzofsky's team showed that T cells present locally in the mucosa could protect against HIV in a mouse model. They translated their work into rhesus macaques and showed that intrarectal immunization was much more effective than systemic administration against infection with the species' equivalent virus, SIV. But, intrarectal immunization was criticized as impractical.

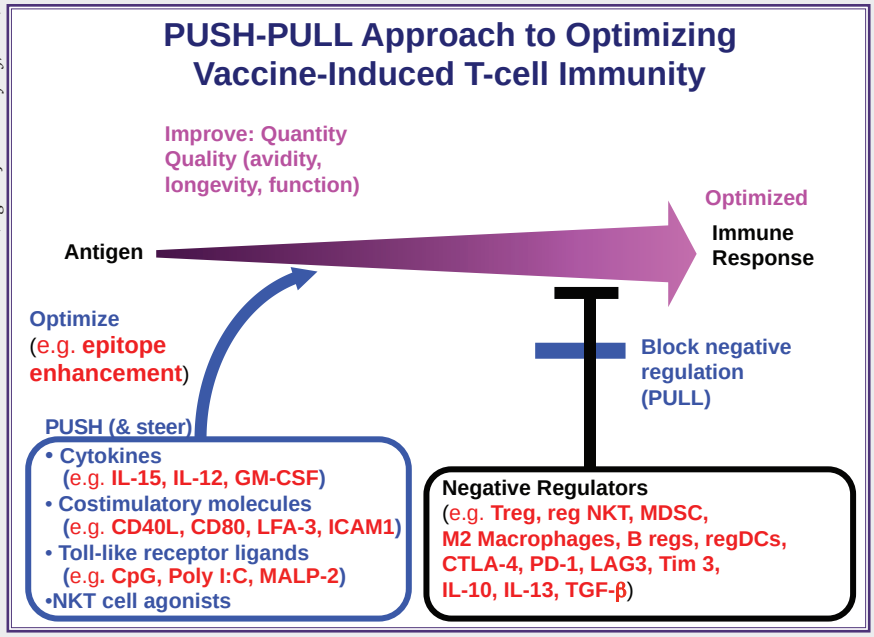
Searching for a new approach, Berzofsky's team worked with Nanotherapeutics to develop a nanoparticle vaccine that could encapsulate HIV epitopes, shield them from interaction with the stomach or small intestine, and only gradually dissolve them in the large intestine to release the vaccine into the colon.

Sui is now translating that research from mouse to macaque. In the first cohort, the vaccine clearly protects the animals from rectal challenge with SIV. The second cohort is still under study. "If everything goes as expected, we calculate the efficacy as 40–50 percent protective," said Sui. "For a vaccine to be considered for potential use in man, we will need to show 60–70 percent efficacy. Our plan is to add a topical microbicide."

Meanwhile, the team published a paper earlier this year in *Nature Communications*, in which they demonstrated that vaginal immunity to HIV could be induced locally in the vaginal mucosa. "This goes against textbook dogma, which states that dendritic cells must pick up an antigen and carry it through the lymphatic system to induce immunity in T cells at the lymph nodes, which then circulate back through the blood stream," said Berzofsky. "The vaginal mucosa has no organized lymphoid tissue."

Thus, their work on the HIV vaccine has led them back into the basics of mucosal immunology.

"Our laboratory is multi-dimensional: on the one hand, we have cancer, on the other, HIV. Then, we have basic research, which we translate into animal models and then into the clinic," concluded Berzofsky. "Rather than spreading ourselves thin, each of our activities reinforces and feeds the others."



(Figure: J. A. Berzofsky, CCR)

To learn more about Dr. Berzofsky's research, please visit his CCR website at http://go.usa.gov/3JwJW.

A Surgeon's View of Prostate Cancer

Robert Reiter, M.D., M.B.A., is a Professor of Urology and Molecular Biology, Director of the Prostate Cancer Treatment and Research Program, and Director of Urologic Research at the David Geffen School of Medicine at the University of California, Los Angeles (UCLA). After studying medicine and completing a residency in general surgery at Stanford University, Reiter went to Baylor College of Medicine for training in urology. Reiter undertook additional fellowship training in urological cancer in CCR's Surgery Branch; he also conducted postdoctoral research focused on the molecular biology of prostate and kidney cancer with W. Marston Linehan, M.D., Chief of the Urologic Oncology Branch. In addition to his clinical practice, Reiter runs a large basic and applied research program in prostate cancer. He is currently the Principal Investigator of UCLA's prostate cancer Specialized Program in Research Excellence (SPORE), which has been successfully renewed twice. In 2007, he completed his business degree at UCLA, and he has been involved in three start-up companies to translate his discoveries into improved outcomes for patients with prostate cancer.

Looking back, I thought I wanted to be an academic clinician-scientist and thought I needed the intellectual stimulation of an academic setting, but I was not sure what kind of research I wanted to do or even if I wanted to do research. I had done three years of clinical training at Baylor, so I stepped out and explored something different. I had two fantastic years at NCI.

I trained at NCI on the recommendation of our chair at Baylor, who had also spent time there. I was looking to explore research and find what I wanted to do. In the clinical service, we were doing immunotherapy and taking care of patients with an inherited form of kidney cancer, Von Hippel-Lindau (VHL) disease. The lab was trying to clone the *VHL* gene at the time, and I worked on number of projects, including one of my own to look at mutations in the *p53* gene in kidney cancer.

Personally, I found the link between what I was doing clinically and what we were doing in the lab rewarding; and that is what got me interested in an academic medicine career. It set me on a course that I continue today.

Markers of Success

My research remains highly translational; it is about taking insights from the clinic into the lab, whether from the operating room or patient management. Today, I spend about 60 percent of my time doing surgery and taking care of patients, and about 40 percent of my time on running the laboratory and administrative work. Our lab has been focused on identifying new therapeutic and imaging targets in cancer. We have developed a series of different antibodies against prostate cancers, aimed both at therapies and at imaging for surgery or disease monitoring.

We have a definite interest in cancer stem cells. In the late 1990s, we first identified prostate stem cell antigen (PSCA) as a cell-surface marker overexpressed in prostate cancer. Early on, I wrote a number

(Photo: Courtesy of R. Reiter)

Robert Reiter, M.D.

of papers with the hypothesis that prostate cancers arise from stem cells in the basal cell layers, and, interestingly, the field has evolved to suggest that this is actually true! We are still interested in understanding the evolution of differentiated cell types from these stem cells, in particular, the evolution into neuroendocrine tumors.

In 2010, we published a paper in *Nature Medicine* in which we showed that N-cadherin, a mesenchymal cadherin associated with epithelial-to-mesenchymal transition (EMT), was reproducibly upregulated in several models of castration-resistant cancer. We showed that the ectopic expression of N-cadherin is sufficient for converting androgen-dependent prostate cancer into invasive, metastatic, and castration-resistant prostate cancer in animal models, and that these effects can be inhibited by N-cadherin-specific antibodies. We are now trying to understand the role N-cadherin plays in the transdifferentiation process.

> "We have taken several antibodies into clinical trials..."

Surgical Strikes

Prostate cancer can be successfully eradicated through surgery, but a major correlate or predictor of failure is the presence of cancer at the margins of a tumor that is excised. Surrounding the prostate are nerve bundles that control the bladder, urethra, etc…, making surgery particularly challenging. You are always trying to split hairs: preserving normal function while getting cancer out. It is almost impossible to do that perfectly. If we could see the edges of the cancer, we could do a better job of excising it.

In order to address this problem, we are engineering antibodies to PCSA that are conjugated to different fluorophores that could help us visualize the cancer cells in the operating theater. In addition, we have developed different animal models that can replicate the kinds of problems we see in the operating room in order to test our antibodies.

Over the years, my closest collaborator has been Anna Wu, Ph.D., Professor of Molecular and Medical Pharmacology at UCLA. She has a background in radioimmunotherapy. I have clinical insight into the problems that can be addressed through antibody targeting; she has expertise in antibody engineering and radiobiology. My lab does the target identification and animal modeling; her lab reengineers the antibodies.

We have taken several antibodies into clinical trials and even started a few companies. My first commercial experience was with an antibody company spun out of my department; it licensed one of the antibodies that was developed in our laboratory. Then I went to business school, and eventually started a company in 2007 to develop a prostate imaging agent and an imaging agent to track the immune system during immunotherapy. The company is venture backed and currently testing agents in the clinic. We have also started a virtual company to commercialize some of our newer antibodies. The whole purpose of my research is to try and make a difference for patients, and, in my opinion, start-up companies are vital channels for translation into the clinic and a way to maintain some control over the translational process once it leaves academia.

Surgeons and Science

If you look at the evolution of different fields across medicine, success has depended on discoveries that emanate from those fields. Urology has seen these successes many times, whether in the treatment of kidney stone diseases or the management of prostate cancer by castration (for which Charles Huggins, M.D., was awarded the Nobel Prize in Medicine).

Surgical fields have not been as adept at recruiting, fostering, or training clinician-scientists. I was just at a molecular biology course sponsored by the American Association for Cancer Research this summer and 90 percent of the students were medical oncologists. Research is a more established career path in medical oncology, so it is not surprising that most of the major advances in cancer treatment and biology (with notable exceptions) are coming from medical oncology or basic science, and less so from urology and even surgery. Add to that the economics of our time, which result in residency programs dropping their research year requirement, and research experiences like I had at CCR are fewer and farther between. I hope this trend reverses because the future of these fields depends on not just clinical practice but on research conducted by those practitioners.

Radiation Therapy in the Modern World

Deborah Citrin, M.D., Senior Investigator in CCR's Radiation Oncology Branch, came to the NIH in 2001 as a radiation oncology resident after completing her medical training at Duke University. She continued her training through the CCR Clinical Investigator Development Program, which is specifically designed to aid in the transition between a mentored position and that of an independent investigator. In 2007, Citrin became a Tenure-Track Investigator and was awarded tenure earlier this year. Throughout her years of training and service, Citrin has been committed to improving the efficacy of, and reducing the complications that arise from, one of the most effective treatments we have for cancer: radiation.

The goal of all of my work is to improve radiation treatment for patients who have cancer, either by further sensitizing tumors to radiation damage or protecting healthy tissue from it. Radiation therapy works by bombarding cells with highly energetic electromagnetic waves or particles, either from an external source or an internally placed radioactive source. The radiation can damage DNA and other molecules in all cells, but rapidly proliferating cells like cancer are most vulnerable to destruction. In one sense, radiation was one of the first targeted treatments for cancer; like surgery, it is localized to a particular treatment area. And, thanks to improvements in the underlying technology, we have had impressive advances in radiation treatment delivery over the last century, such that we are able to better target tumors and spare most normal tissue. Nonetheless, damage to healthy tissue is still a concern whenever radiation is applied.

(Photo: R. Baer)

Radiation Therapist Dramane Niambele and Deborah Citrin, M.D., prepare a patient in a TomoTheraphy unit.

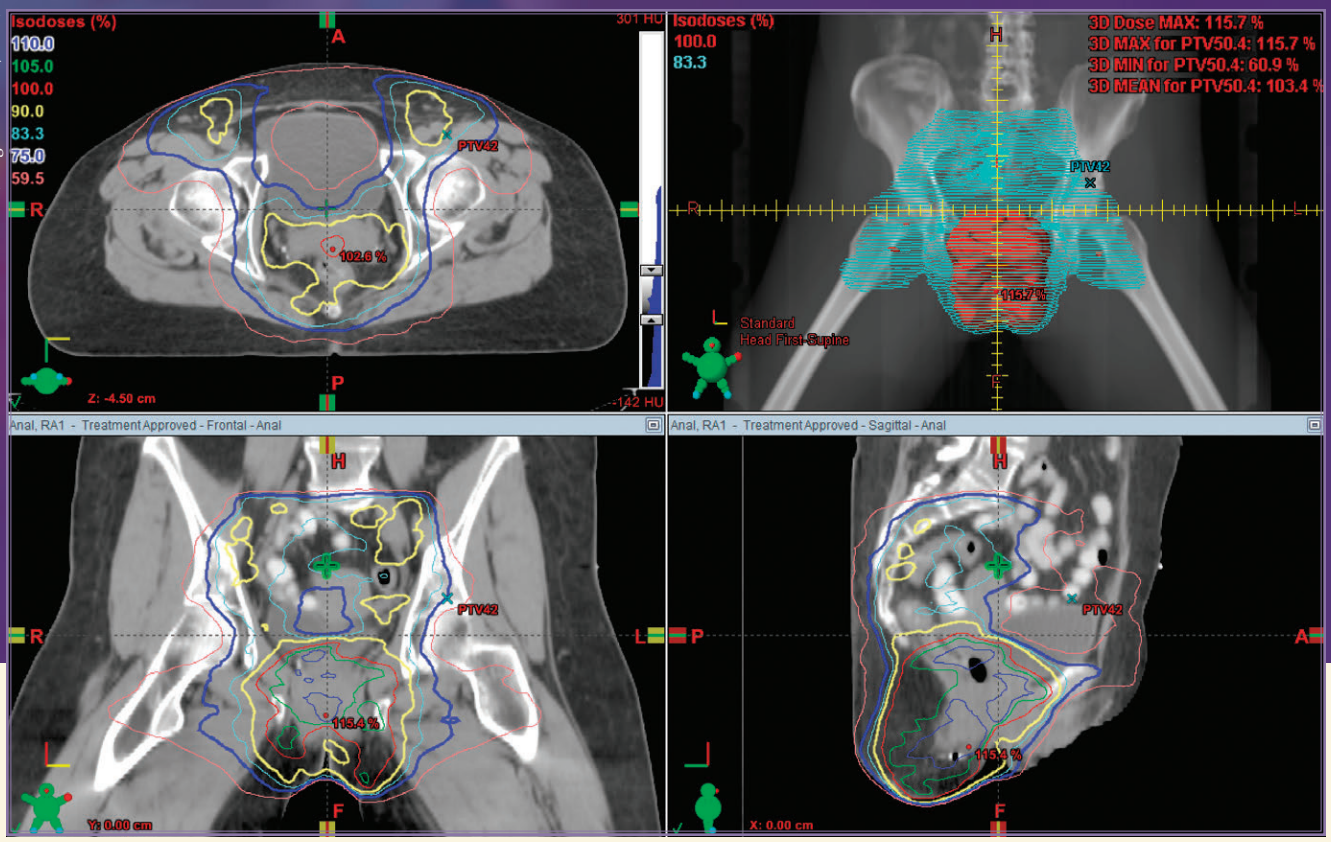
Protecting the Healthy

Radiation fibrosis is a scarring, which can occur in organs like the lungs or the skin, causing tremendous complications, morbidity, and even mortality. It has often been considered an irreversible side effect. We have studied radiation fibrosis extensively in the laboratory, with the goal of developing therapeutics. Unlike acute radiation injury, this kind of scarring can happen months to years after treatment. Thus, we have to develop longer-term models in animals and cell cultures than typical cancer models. It can take four to six months to study, treat, and follow the progression of fibrosis in mouse models, making these experiments time consuming and expensive.

We've known since the 1960s that inflammation is very important in radiation injury; you can visualize it in stained tissues. Because of its involvement in inflammation and fibroblast activation, many in the field have focused on TGF-β as a key molecular driver of radiation fibrosis.

Instead of focusing on this single molecule or the late time point at which we see the manifestation of

(Image: D. Citrin, CCR)



Treatment planning images for an anal cancer patient. In the upper right panel, a rendering of the two tumor target volumes (red—high risk, blue—intermediate risk) are outlined. In the other panels, axial (horizontal), coronal (frontal), and sagittal (median) images of the treatment planning computed tomography (CT) show the distribution of radiation dose. Each line represents a dose level (dose key in percent in the upper left hand corner).

injury, we have taken a basic approach to understanding the chronology and molecular pathways that are activated, beginning at the time of exposure to radiation, then into the latent phase in which we do not see any manifestation of injury, and finally into inflammation and fibrosis. Through this approach, we hope to find novel pathways and processes that may be amenable to intervention before the damage is irreversible.

We started by performing microarray analyses of gene expression in our mouse models. We noted that the pattern of gene expression in irradiated mouse tissues bore a strong similarity to the gene expression in tissues of older mice, almost as though radiation was aging the tissue. This finding prompted us to look for stem cell senescence in the irradiated tissue. And, in fact, we saw that radiation caused stem cell senescence, reduced proliferative potential, and lower numbers of newly differentiated cells.

We have also found that the senescent cells are capable of promoting senescence in otherwise healthy cells through a paracrine process involving the secretion of cytokines or other protein factors. Thus, it is possible that radiation damage to a few cells goes on to induce senescence in additional stem cells in a positive feedback cascade. Intervention in this process could stop the progression of the fibrosis.

We have identified several agents that are capable of preventing both senescence and fibrosis in our models, and we have many more targets of interest based on the patterns of gene expression we observed. We have a few agents that may be useful in the treatment or prevention of fibrosis, if they are delivered to patients. For some of these studies, we have been working closely with James Mitchell, Ph.D., Chief of CCR's Radiation Biology Branch.

For example, we recently found that plasminogen activating inhibitor-1 (PAI-1), known to be important for fibrosis through the stabilization of fibrin, is a critical mediator of senescence. Our collaborator, Mary Jo Mulligan-Kehoe, Ph.D., who was at Dartmouth University until her retirement, developed a truncated PAI-1 protein that essentially prevents the signaling cascade activated by PAI-1, reducing fibrin stabilization, fibrosis, and senescence.

Currently, we are trying to understand the best timing for delivering these agents because we would like to intervene as early as possible, while also restricting the intervention to those patients most likely to develop the complication. A mouse's lifespan is much shorter than that of a human; how do we translate

> "We recently found that plasminogen activating inhibitor-1 (PAI-1), known to be important for fibrosis through the stabilization of fibrin, is a critical mediator of senescence."

the effects we are seeing in our models onto the human timescale?

As a different approach to mitigating radiation injury, we have also looked at mesenchymal stem cells from the bone marrow. These are multipotent cells with anti-inflammatory properties. We have found that just a single dose of these cells administered systemically to our mice is sufficient for dramatically reducing fibrosis. A single infusion completely changes the biology of the tissue. The cells migrate to the site of radiation injury, altering the inflammatory response. We have now demonstrated this effect in both the skin and lungs of our animal models. It would be relatively straightforward to use donated cells to treat patients based on a similar strategy. For this work, we have been collaborating with Pamela Robey, Ph.D., Chief of the Craniofacial and Skeletal Diseases Branch, National Institute of Dental and Craniofacial Research, and Co-Coordinator, NIH Bone Marrow Stromal Cell Transplantation Center.

Skin damage is another area of interest for us. We recently completed a trial of a topical radioprotector for radiation dermatitis in patients with anal cancer. MTS-01 is a formulation of tempol, a nitroxide that scavenges free radicals, which Mitchell studied as a radioprotector in animal models. It is in some ways selective to normal tissues as compared to tumor tissues, which makes it a really interesting compound. It can protect from lethal total body exposure and radiation-induced hair loss. In the treatment of anal cancer, radiation induces a high risk of skin toxicity that can be so severe that patients need to be hospitalized for pain relief. Newer technologies, i.e., intensity-modulated radiation therapy (IMRT), have mitigated that toxicity; however, we still see substantial redness, irritation, and small areas of skin peeling or desquamation.

MTS-01 is easy to use; it is applied once daily just before radiation treatment. We have completed the trial and seen some very promising responses in individual patients (see "Finding the Right Care"), but we do not have the final data yet to know how well MTS-01 worked overall. We are still following patients to study the long-term differences in toxicity. The compound is currently being developed in a randomized clinical trial, which includes hundreds of patients, for a similar indication, so hopefully its value will become apparent.

> "My focus has moved toward studying cases of resistance to radiation in order to allow us to rationally select new agents for study as sensitizers."

Strengthening the Attack

In addition to looking for ways to protect healthy tissue from the effects of radiation damage, I have been looking for ways to sensitize tumor cells so that the same or lower doses of radiation are more effective in destroying diseased tissue. For example, we have known that the Ras pathway is activated rapidly after radiation and that mutations in the Ras pathway are associated with radiation resistance. So, several years ago, we decided to test the hypothesis that inhibiting one of the signaling molecules downstream of Ras, i.e., MEK with AZD6244/selumetinib, would make tumors more vulnerable to radiation. Following up on favorable results in a variety of cell lines, we went on to look at combinations of chemotherapy, radiation therapy, and the MEK inhibitor in animal models, with an eye towards clinical translation. Ultimately, this led us to a clinical trial of this combination for rectal cancer. A newer generation of MEK inhibitors is now being tested in combinations at other institutions.

Multiple investigators at CCR and elsewhere have also identified other pathways implicated in radiation resistance, such as AKT, mTOR, and DNA repair, and developed radiation sensitizers. We learn more about the biology of irradiated tissues at the same time as we identify sensitizers. For example, if we find that inhibiting a pathway enhances the effect of radiation on a tumor, we may find other tumors or cell lines that are not similarly sensitized. These tumors can teach us a great deal about the mechanisms of resistance to the sensitizer. More importantly, perhaps, is that we can identify biomarkers of efficacy, predict more accurately prior to treatment which tumors may be sensitized with the combination, and

> "...we have an excellent curative track record with radiation..."

identify redundant pathways that may also be targeted simultaneously to allow effective sensitization. Unfortunately, for every agent we identify as a sensitizer, there are many agents that are found to be ineffective in this regard, despite sound biologic rationale. Although we can identify agents that sensitize tumors in this fashion, there is a great deal of interest in using alternative methods to identify candidate radiosensitizers. My focus has moved toward studying cases of resistance to radiation in order to allow us to rationally select new agents for study as sensitizers.

In my clinical work as a radiation oncologist, I specialize in genitourinary malignancies. In prostate cancer, for example, we have an excellent curative track record with radiation, but a small subset of patients develop recurrences locally, distantly (metastatic), or both locally and distantly. We do not know why patients with similar cancers, i.e. identical Gleason scores and PSA levels, can have such different outcomes.

> "My goal is to make radiation therapy safer and more effective for those who need treatment."

I felt we needed a more systematic approach to generating candidate targets for radiation sensitization. So, we opened a protocol to collect and analyze prostate cancer tissue, with the goal of determining which pathways are activated in tumors that are subsequently resistant to radiation compared to tumors that are not. By taking biopsies before radiation therapy and if a patient fails radiation therapy, we will hopefully gain insight into the abnormalities in particular tumors that resulted in radiation resistance and, ultimately, be able to more rationally select potential sensitizers. Eventually, if we can validate our observations, we will pursue interesting targets in the laboratory by studying them initially in cell lines. This work is truly a collaborative effort, relying on my colleagues Peter Choyke, M.D., Director of CCR's Molecular Imaging Program; Brad Wood, M.D., Director of the NIH Center for Interventional Oncology; and Peter Pinto, M.D., Investigator in CCR's Urologic Oncology Branch. Hopefully, this work will guide care in the future by allowing us to more rapidly predict response to treatment or perhaps to identify patients who need more aggressive treatment after radiation.

In the Clinic

I typically spend at least one day per week as the attending physician in the Radiation Oncology clinic. My patients include those who are on protocols that I have initiated as well as those who are on other protocols within NCI and even NIH. Our branch provides radiotherapy for an increasing number of clinical trials initiated in other branches and institutes. For example, as the field of immunotherapy has matured, there has been a strong interest in combining stereotactic body radiation to induce tumor necrosis and trigger the subsequent response of the immune system to tumor antigens. We also provide radiotherapy for a substantial number of transplantation protocols.

(Photo: R. Baer)

Deborah Citrin, M.D.

Over half of all patients with cancer will have radiation at some point; it is a major cornerstone of oncologic care. Although I sincerely wish that we had effective cures that did not require my treatments, I do not see that changing in the near future. We may use radiation differently than we do today, but I suspect that combination approaches utilizing radiation will be a mainstay of therapy for a long time to come. My goal is to make radiation therapy safer and more effective for those who need treatment.

To learn more about Dr. Citrin's research, please visit her CCR website at http://go.usa.gov/3J789.

Finding the Right Care

Trained as a registered nurse and with a doctoral degree in public health, Jane D. is no stranger to the U.S. health care system. But, when she found herself facing a diagnosis of anal cancer in 2013, she felt adrift.

"An initial conversation with the surgeon on my case was scary, but also encouraging and reassuring. He said that the chances of successful treatment were high," Jane said. "But, neither I nor anyone in my immediate family has had cancer; it was new territory for me."

At the time, Jane was working 60 hours per week in a demanding career; she felt overwhelmed. She started searching the Internet and found the American Cancer Society website, with a listing of helpful resources.

"Then it occurred to me that I might look at the NIH. It seemed like a long shot, but I found this trial led by Deb Citrin. I e-mailed her and heard back within 15 minutes. I was amazed by her responsiveness."

Jane still needed to have work-ups done to find out if she would fit the trial criteria. She and her husband had a meeting with Citrin and her team, who walked them through the process. She would need to go to the NIH five times per week for radiation treatments and get chemotherapy in two separate treatment sessions. The trial included application of a topical drug (MTS-01) to reduce the complications of skin lesions resulting from intense radiation. "Deb talked about the side effects and what I could expect in terms of being able to work, in far more realistic terms than what I had originally been told," Jane said.

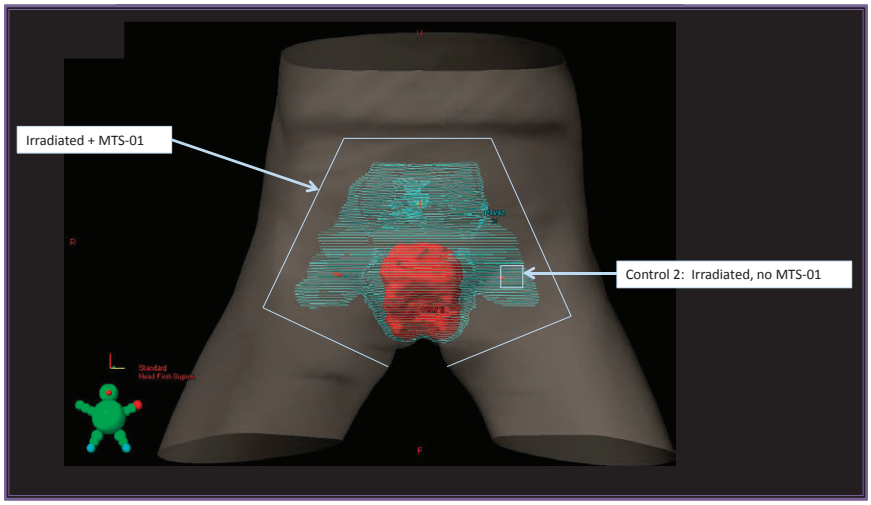
Indeed, the treatment was grueling and Jane was unable to keep up her busy work schedule. The radiation treatment to the pelvis area made it difficult for her to walk, and her red blood cell counts were very low. "It was a process during which I just got weaker and weaker," Jane said. "But I was able to complete the treatment, and, in that sense, the topical drug seemed to work."

Jane's case was followed with quarterly scans at the NIH during the first year and then through her HMO in the second year. She has been cancer-free since the treatment.

Cancer is a traumatic event, but Jane struggled particularly with her diagnosis. "In certain contexts, outside the circle of my friends and family, I didn't really want to say that I have anal cancer; I would say I have colorectal cancer, as much for the other person as myself. There was just a dimension of embarrassment."

Jane volunteered herself to be a contact for others going through Citrin's clinical trial and has been in touch with patients who have come after her. "This is a disease where you can go to the Internet and find a lot of people who have gone through treatment or are in treatment making comments, but I don't think that's the healthiest way to get information.

"I felt the NIH offered such tremendous expertise not only from Deb Citrin and her team, but also from my medical oncologist team. And, the interpersonal warmth, humor, and 'down-to-earthness' of the people that were caring from me was just amazing. I kept thinking that this is the way health care should be in the U.S. for everybody, and it makes me sad that it isn't," she said.



(Image: D. Citrin, CCR)

Image depicting the application area for the topical drug, MTS-01. Red indicates the tumor in the anal canal with a small margin. This area will receive the highest dose of radiation. Blue indicates an area that contains lymph nodes that may be contaminated with tumor. This area will receive a lower dose of radiation. The pale blue lines demarcate the region where the most severe skin reaction is expected and where MTS-01 will be applied.

CCR connections is available online at http://home.ccr.cancer.gov/connections.

Websites with More Information about CCR

Center for Cancer Research
http://ccr.cancer.gov

Office of the Director
https://ccr.cancer.gov/office-of-the-director

CCR News
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CCR on Social Media
https://ccr.cancer.gov/social-media

Career Opportunities
https://ccr.cancer.gov/positions

Training Opportunities
https://ccr.cancer.gov/training-office-of-training-and-education

Patient Information on Cancer and Clinical Trials

Open NCI Clinical Trials
http://www.cancer.gov/clinicaltrials/search

How to Refer a Patient
https://ccr.cancer.gov/physicians

NCI Cancer Information Service
http://www.cancer.gov/aboutnci/cis
1-800-4-CANCER (1-800-422-6237)

CCR Clinical Cancer Trials in Bethesda, Md.
https://ccr.cancer.gov/clinical-trials-search-start

Additional Links

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http://www.cancer.gov

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