Giovanni Armenise-Harvard Foundation
11th Annual Symposium

Genetic and Epigenetic Regulatory Mechanisms in Human Disease

Hyatt Regency Newport Hotel and Spa
Newport, Rhode Island
June 8-11, 2007

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About the Symposium

Although most educated people view DNA as the molecule solely responsible for inheritance, a more complex and nuanced story unfolded during the 11th Annual Symposium of the Giovanni Armenise Harvard Foundation. Held in Newport, Rhode Island, on June 8-10, 2007, this year's theme was "Genetic and Epigenetic Regulatory Mechanisms in Human Disease."

Anyone tall enough to peer through a microscope can see that fat, hemoglobin-rich blood cells don't look anything like neurons with their long, spidery arms. Yet the same DNA is packed into the nuclei of these wildly different cells. So DNA, scientists now say, is not all there is.

Today's consensus is that structural and functional differences among cell types are determined by "epigenetic" programs that regulate DNA's activity, some of them as marks from environmental assaults, some a form of memory. What makes these proteins and small molecules so important is their capacity to change genetic expression without altering DNA sequence itself.

Epigenetic changes turn an embryonic stem cell into a specialized adult cell and transform normal cells into tumors, according to Whitehead Institute investigator Rudolph Jaenisch. In his keynote address, Jaenisch said understanding how to program and reprogram different types of cells is one of the most important topics in biology today, because such skills would hold enormous promise for treating the ills of humankind.

Epigenetic "reprogramming" is a key step for creating stem cell therapies for cancer and progressive disorders such as Parkinson's disease, yet these efforts are ensnared in ethical and political complications. Two days before the symposium began, the House of Representatives voted to expand government-supported stem cell research, a piece of legislation destined for Presidential veto despite protests from patients and families. On the same day, *Nature* published important stem cell findings from Jaenisch's lab. Elsewhere, cancer patients were participating in clinical trials testing drugs with epigenetic activity, hoping for cures.

These are the real-world complications of the basic science presented by Jaenisch, 18 other speakers and numerous poster presenters at the 2007 symposium. Participants convened at the Hyatt Regency Newport Hotel and Spa on June 8-10, marking the symposium's return to the United States after four consecutive meetings in Italy.

Powerful ties to Harvard science made Newport an appropriate setting for this year's meeting. It was here that Alexander Agassiz, curator of Harvard's Museum of Comparative Zoology, constructed a pioneering marine biology laboratory in 1875. For the next 25 years, his research teams set sail from Newport to probe the physical, chemical, biological and geological features of the great ocean basins.

Agassiz's engineering talents had already made him wealthy in the mining industry; in his second career he used these skills to invent new technologies for gathering specimens and taking measurements in deep water. Long before anyone dreamed of

unmanned submersibles or fiber optics, he made observations about star fishes, sea urchins and other marine creatures that are still cited today.

The contemporary quest to understand how epigenetic factors control genetic information is as challenging as deep-sea exploration was for the Victorians. Yet progress is being made. Some presenters described how epigenetic factors, such as chromatin packaging and methylation, establish a cell's identity yet leave it susceptible to change. Others focused on epigenetic functions as a way of recording what happened to one generation and passing that memory to the next.

Sixty participants accepted the invitation to this year's symposium. Scientists represented Harvard Medical School and its affiliated institutions, Massachusetts Institute of Technology, Yale University, nine Italian universities and research institutes and one multinational pharmaceutical company. Foundation President Joseph B. Martin, dean of HMS, presided and President Emeritus Daniel C. Tosteson, former dean of the medical school, attended along with members of the Foundation's Board of Trustees, Scientific Advisory board and Italian Scholarship Advisory Committee.

At their annual meeting, the Board of Trustees voted to create a new Armenise Foundation Chair at HMS. Count Giovanni Auletta Armenise, who could not be present in Newport, joined this decision via telephone from Italy. The Board named Stephen C. Harrison, Director of the Armenise Harvard Center for Structural Biology, as the first holder of this endowed chair. Harrison's appointment was announced to symposium participants by Dean Joseph Martin.

The program featured talented young scientists who have benefited from Foundation programs at HMS and in Italy. Four winners of HMS Junior Faculty Grants participated in the symposium, including two of three researchers honored in 2007. They were joined by seven recipients of Career Development Awards, which enable Italians to return home and establish their own laboratories after completing post-doctoral training abroad. The Dulbecco Telethon Institute also makes research grants for this purpose, and the Foundation invited Telethon-supported epigenetics researchers to join this year's symposium and meet potential collaborators.

Also on hand were two Italian science journalists, the latest recipients of the annual Science Writer Fellowships that enable Italian reporters to research stories of their choosing at HMS and participate in the symposium.

Keynote Address

Pluripotency, Epigenetic Reprogramming and Embryonic Stem Cells
Rudolf Jaenisch
Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

Thirty years ago, young Rudolph Jaenisch became famous beyond his years by creating the first transgenic laboratory animals in history. He showed that when Moloney

leukemia virus was injected into early mouse embryos, viral gene sequences not only integrated into the DNA of these animals but also passed to their offspring.

On the day before the Armenise-Harvard Symposium began, Jaenisch made headlines for a different reason: his group and two other teams revealed that they had transformed adult skin cells into pluripotent embryonic stem cells. These cells were incorporated into different tissue types and could be inherited by offspring. Importantly, these groups had created embryonic stem cells without running afoul of controversies about using eggs or destroying embryos.

In the Jaenisch lab, this accomplishment builds on more than two decades of therapeutic cloning research. Using mice as a model, the researchers substitute DNA from a fibroblast for the original DNA in an unfertilized egg, which is then grown into a blastocyst to generate stem cells for animal "patients." Although Jaenisch believes this approach – known as somatic cell nuclear transfer, or SCNT -- could be successful in human patients, he said it is "not financially feasible, it is too difficult to obtain enough eggs, and there are many ethical barriers."

In the course of his nuclear cloning experiments, Jaenisch was intrigued by the possibility of resetting the epigenetic hallmarks of an adult donor nucleus to those of an embryonic cell. To accomplish this, Jaenisch reckoned he would need to understand how embryonic stem (ES) cells replenish themselves while still being able to differentiate into nearly any type of adult cell.

Fortunately, his team and others have learned a great deal about the molecular circuitry of pluripotency and self-renewal, making it possible to differentiate pluripotent cells into various specific cell types and to take somatic cells backward in time, restoring elasticity they possessed at an earlier developmental stage.

Japanese researcher Shinya Yamanaka and his colleagues at Kyoto University were engaged in a similar quest, and in August 2006 they reported that activating genes for four transcription factors in skin cells from adult mice had returned those cells to a pluripotent state. Unfortunately, these cells were not as flexible as real embryonic stem cells and could not be used to generate live mice.

Jaenisch's lab set out to refine these experiments, and after screening various transcription factors for genome-wide activity the researchers decided to focus on Oct4 and Nanog. These factors are active *only* in fully pluripotent cells, where they stimulate an armada of genes that retain elasticity and repress genes controlling differentiation. The team devised a clever method for collecting cells successfully reprogrammed by active Oct4 and Nanog, then tested their capacity to behave like embryonic stem cells.

The reprogrammed cells contributed to every tissue type after being injected into early-stage embryos and these embryos developed into live mice. When these mice were bred, descendents of the reprogrammed cells were detected in the next generation. Additional experiments were performed as well, and the altered cells were indistinguishable from embryonic stem cells on all counts.

When it comes to cell-based therapy for human disease, however, "we are not there yet," Jaenisch cautioned. The retroviral vectors used to reprogram the cells are oncogenic and could not be ethically used in humans, scientists don't know whether the

transcription factors that return mouse fibroblasts to pluripotency would work for humans, and better ways are needed to separate reprogrammed cells from adult cells that did not succumb.

Ultimately, findings like these could propel cell-based treatments for human disease over scientific and ethical hurdles associated with using human embryos for therapeutic purposes. For now, however, these findings are “preliminary and proof of principle,” Jaenisch said.

Presentations

Systems Analysis of Human Mitochondrial Disorders

Vamsi K. Mootha, MD
Department of Systems Biology, Harvard Medical School,
Center for Human Genetic Research, Massachusetts General Hospital, and the Broad Institute of MIT and Harvard

As soon as mitochondrial DNA became “the first human genome ever sequenced,” back in 1981, scientists recognized that it encoded only 13 complete proteins. Since then, about 50 human diseases and syndromes have been attributed to mitochondrial abnormalities. These diseases are difficult to diagnose and so far there are no curative therapies for any of them.

What’s fascinating “is that most clinical mitochondrial patients do not have lesions in the tiny mitochondrial genome – only about 15 percent do,” Vamsi Mootha said. “The rest have problems with nuclear genome proteins that regulate mitochondrial activity.” It appears that mitochondria draw nuclear proteins into their orbit, and the new recruits can cause problems.

Mootha’s laboratory uses human genome sequence data, microarrays, and computer algorithms to systematically link mitochondria to human diseases. Several years ago, Mootha and his collaborators used these tools to identify the mutation responsible for Leigh syndrome, French Canadian type. This lethal disease was tragically common among children in a remote population in Quebec, affecting one in every 2,000. Mootha’s work led to a prenatal test now being used by families at risk.

The Mootha team has since created a new system, called “Maestro,” that is helping them construct a complete protein atlas, or proteome, for mitochondria. This system integrates data from eight different protein screening methods, cross-checking one against the others, to pinpoint proteins active in mitochondria. So far, it appears that about 1,500 proteins function in this tiny but industrious organelle.

The team is using this “parts list” to look for specific gene defects associated with rare mitochondrial disorders, and the search is going well. Mootha collaborated with Massimo Zeviani’s laboratory at the National Neurological Institute in Milan to change misconceptions about hepatic mitochondrial DNA depletion syndrome, a disorder that had been attributed to peroxisome malfunction.

The real culprit is a defect in MPV17, which encodes key components of the mitochondrial membrane. In a poster presentation at the Symposium, Antonella Spinazzola described the impact of this mutation in families studied in Italy and on a Navaho reservation in the United States, as well as in a mouse model of disease.

Ongoing work in the Mootha lab is aimed at achieving a systems-level understanding of mitochondria that will improve diagnosis and treatment for many other, more common human diseases.

Targeting the B-cell Antigen Receptor in B Lymphoma Cells: Lessons from Mouse Models

Stefano Casola
Foundation IFOM, the FIRC Institute of Molecular Oncology, Milano, and the CBR Institute of Biomedical Research, Harvard Medical School

The incidence of non-Hodgkin (NHL) lymphomas has doubled over the past two decades, advancing it to fifth place on the list of common cancers among men and women. In the United States, more than 63,000 new NHL cases are diagnosed each year and nearly 20,000 people die. This increase is unlikely to level off because these lymphomas are associated with rising rates of infectious diseases, autoimmune disorders, and toxic environmental exposures.

Clinicians need new therapies for NHL patients, 80 to 90 percent of whom have B-cell tumors that originate in lymphoid tissue. Stefano Casola uses mouse models to peer inside tiny factories where some B cells are programmed to stand watch against invaders. While normal sentries die at the end of their shift, others live too long and turn traitorous. A surface complex called the B cell antigen receptor (BCR) is important for their differentiation, proliferation and life span. Mature B cells undergo apoptosis when BCR is lost after several weeks.

In contrast, functional BCR expression persists in most types of NHL cells, including Burkitt's, follicular lymphoma and diffuse large B-cell lymphoma. Casola's team set out to understand the role of the BCR in these cancer cells, and to see what would happen if they manipulated this complex in cells from mice with the equivalent of Burkitt's lymphoma.

A human c-MYC oncogene was used to induce tumors that look like human Burkitt's lymphoma under the microscope and have the same cell surface markers. In a series of *in vivo* and *in vitro* experiments, the researchers selectively blocked the activity of BCR and other genes thought to be involved in B cell survival, proliferation and differentiation.

When the researchers disabled BCR, B-lymphoma cells isolated from independent primary tumors rapidly died, both *in vitro* and *in vivo*. Compared with BCR+ cells, the BCR- ones were three times more likely to undergo apoptosis. For reasons that are poorly understood, the cells suddenly deprived of BCR died only when BCR+ cells were present. It's possible that having BCR is an advantage only when BCR- cells are also present and competing for nutrients, a situation Casola characterized as "restrained growth." He sees preliminary evidence that BCR+ cells may be better able to take up glucose in this setting, enabling them to out compete BCR- cells.

Casola and his team are now looking for highly conserved steps in the BCR signaling cascade regulated by epigenetic factors such as histone modifiers. They may be able to hasten the death of malignant B cells if they can identify such interim steps.

Tumor Suppressor Activity of the Histone Acetyl-Transferase Tip60

Bruno Amati
Department of Experimental Oncology, European Institute of Oncology (IEO),
IFOM-IEO Campus, Milano

Drunken teenagers challenge one another to jump from a dangerously high railroad trestle. When one makes a move toward the edge, he's unlikely to leap if he is restrained by a friend on each side. But if there is only one friend dragging him back, the danger increases that he'll break free and take the plunge.

Apparently the same is true for cancer cells, which are torn between the drive to proliferate wildly and the more sober impulse to stop dividing and undergo normal apoptosis. Having two copies of tumor suppressing genes on hand reduces the risk of malignant transformation; but if one copy is lost – so that the tumor cells become "haplo-insufficient" – anything can happen.

Bruno Amati's lab investigates epigenetic regulation of humor development, and his symposium presentation focused on Tip60, an acetyl-transferase that modifies chromatin structure and influences transcription factors that either promote or suppress tumorigenesis. Tip60 has been characterized as a haplo-insufficient tumor suppressor in human breast cancer, and Amati uses mouse models to probe its activities in detail.

In these models, the Myc oncogene promotes B cell lymphoma while paradoxically activating DNA-Damage Response (DDR) signaling, a type of tumor suppression. Pre-tumoral B-cells of young Em-myc mice showed a sustained DDR as judged by phosphorylation of ATM, H2AX, Chk1, Bid and p53 (Ser 15).

When Em-myc transgenic mice were crossed with heterozygous Tip60 knockout animals, DDR was severely impaired and tumors grew faster, even though there was no significant change in pre-tumoral B-cell expansion, S-phase or apoptosis. The researchers were surprised to see that the wild-type Tip60 allele was never lost in lymphomas, but was duplicated instead. They concluded that Tip60 acts as a haplo-insufficient tumor suppressor during early tumor development, but not when cancer is more advanced.

Myc is a strong inducer of the ARF-p53 tumor suppressor pathway. Lymphomas arising in Em-myc p53+/- mice lose the remaining p53 allele, except when this loss of heterozygosity (LOH) is blocked by secondary mutations that impinge on p53 activity and/or apoptosis. In lymphomas arising in Em-myc p53+/- Tip60+/- mice, however, secondary mutations did not prevent p53 LOH. A similar observation was made for ARF LOH in Em-myc ARF+/- Tip60+/- mice. Selective pressure to lose the ARF-p53 pathway in these mice implies that Tip60 haplo-insufficiency does not bypass ARF-p53 function, even though p53 phosphorylation is impaired, Amati said. Redundant mechanisms for DDR must be suppressing tumor growth by other means.

Finally, the haplo-insufficient tumor suppressor activity of Tip60 appears to kick in only when oncogenes are active in incipient tumor cells. Like the boy who pulls a friend back from the edge, restraint is needed only when danger threatens.

Sonic Hedgehog—Proteoglycan Interactions in Development and Disease

Rosalind Segal
Department of Neurobiology, Harvard Medical School and Department of Pediatric Oncology, Dana-Farber Cancer Institute

Sonic hedgehog is one of the best known morphogens in biology, and arguably the most colorfully named of the signaling proteins that touch large groups of embryonic cells and guide them to form spinal cords, wings or fingers. Sonic hedgehog (Shh) promotes synchronized growth and specifies cell fate as patterns develop in complex tissues.

Shh is an exceptionally versatile protein that acts on many eukaryotic intracellular pathways. Rosalind Segal examines Shh's interactions with proteoglycans found outside the cells, in the extracellular matrix, and asks how these might be important for oncogenesis. This is a clinically important question because Shh overactivity has been described in some tumors of the brain, skin, breast and pancreas.

In animal models, as in human cells, the Cardin-Weintraub motif in Shh protein binds tightly with heparin-sulfate proteoglycans. When Segal's team introduced mutations that disrupted this binding, the mice had features that were normally patterned but abnormally small. Observations in mice and *Drosophila* indicate that such disruptions interfere with Shh's normal expansion of stem cell pools without affecting morphogenesis.

More recently, the Segal lab has been exploring how Shh-proteoglycans interactions affect what happens during mitosis. Like many other researchers, she's become fascinated by how signals from outside the cell, such as proteoglycans, integrate with intrinsic cell fate programs to regulate cell division. During normal stem cell mitosis, one daughter is like the original – replenishing the stem cell pool – while the other prepares to differentiate. This "asymmetric" division maintains normal growth and development.

In neural stem cells, however, Segal and her colleagues introduced mutations that disturbed typical Shh-proteoglycan interactions and – among other effects – induced *Gli3, Bmi1* and *cyclinD1/2.* Instead of undergoing normal, asymmetric division, these cells divided much faster than usual. Heparin-sulfate proteoglycans localizes Shh to mitogenic niches, and under certain conditions appears to hold the pedal to the metal, speeding up cell division to a malignant degree.

Segal emphasized that this is only a preview of what's to come. "There are more types of proteoglycans in a mammal than there are stars in the sky," she said, and she hopes that genetic approaches to "glycomics" will be able to finger more of the bad actors. And this, in turn, raises the possibility of shrinking tumors – without harming normal development – by blocking specific types of proteoglycans activity.

Cellular and Organism Toxicity in a Drosophila Model for the Polyglutamine Disease DRPLA

Manolis Fanto
Dulbecco Telethon Institute, DIBIT-San Raffaele Scientific Institute, Milano

Although Huntington's is the only polyglutamine (polyQ) disease most people know about, it is only one of about a dozen progressive neurological disorders in this group. What they share is the genetic equivalent of a stutter – relentless repetition of CAG, three nucleotides that encode the amino acid glutamine. This happens in a so-called polyglutamine tract, a part of the gene where multiple copies are normal. When stuttering generates excess copies, the resulting protein no longer plays well with others, clogs nerve cells with deposits that stubbornly resist degradation, and ultimately kills the cells.

Because each disease in this group damages a specific protein and destroys cells in a specific brain region, their symptoms are different. Nevertheless, all the PolyQ diseases worsen with time and share a grim prognosis.

Much of what's known about these disorders comes from experiments using the fruit fly *Drosophila melanogaster*, and Manolis Fanto uses novel flies to investigate dentatorubropallidoluysian atrophy (DRPLA), a disorder caused by PolyQ expansion in the human gene for Atrophin-1. Healthy people have six to 35 copies of the CAG repeat in this gene; DRPLA patients have 49-88.

Although some fly models for DRPLA use human genes or synthetic constructs, Fanto and his colleagues have generated a model by expanding endogenous polyQ in *atro.* They combine gain of function and classic loss of function analysis to unravel the cellular and organism consequences of mutations in *atro* and its partner *fat.* His team analyzes cell degeneration in neuronal photoreceptors and uses lifespan as a measure of overall toxicity.

When researchers induced the fly equivalent of DRPLA by expanding a polyQ tract in atrophin, the fly retina developed abnormal vacuoles and the animals died short of their normal lifespan. New findings indicate that the larger the polyQ expansion the worse the retinal damage, which they attribute to disruption of autophagy rather than induction of apoptosis, Fanto reported. Autophagy normally protects cells by clearing away spent organelles.

Abnormal folding makes excess PolyQ proteins clumsy when they interact with intracellular partners. Atrophin and the gigantic cadherin and tumour suppressor *fat* are part of a neuroprotective pathway, and Fanto's experiments have shown that certain mutations in either one can cause neurodegeneration, killing retinal neurons and abbreviating lifespan. The most surprising finding was that the organism dies faster than a cell degenerates, and that this effect on viability is exerted by glial cells as well as by neurons, Fanto said.

Although these studies are far from complete, they hint that the complexity and variation in polyQ diseases may arise not only from the death of cells in a specific brain area, but to more widespread toxicity affecting the whole nervous system. The next steps are to discover the mechanisms involved.

Targeting Active Genes for Histone Modification in *Drosophila*

Mitzi I. Kuroda
Howard Hughes Medical Institute, Harvard-Partners Center for Genetics and Genomics, Brigham & Women's Hospital and Department of Genetics, Harvard Medical School

Mitzi Kuroda's laboratory focuses on a fundamental question in biology: how do organisms balance gene expression in males and females, when males have only one X chromosome and females have two?

The answer lies in "dosage compensation," she said, adjustments to chromatin structure that operate differently in different organisms. In mammals, epigenetic modifications of histone inactivate genes on one of the X's early in female development; in fruit flies, modifications increase expression of genes on the single X so that they match the output of both female X chromosomes in male embryos.

In *Drosophila,* dosage compensation is carried out by a large ribonucleoprotein complex containing MSL (male-specific lethal) proteins and noncoding *roX* (RNA on X) RNAs. This ribbon-like complex attaches itself only to the male X chromosome, where it alters histone H4 acetylation and encourages transcription of X-linked genes.

The *RoX* RNAs differ dramatically in size and sequence, yet they are functionally redundant and necessary for assembling the MSL complex. Once the complex takes shape, it consists of five MSL proteins: if one is missing or inactivated the male embryo dies.

Researchers have struggled to understand how the MSL complex targets specific sites on the X chromosome where binding will increase gene expression. Kuroda's team used a "ChIP-chip" strategy, which combines microarray technology with chromatin immunoprecipitation, to generate high-resolution images of MSL binding and histone modification. They observed wild-type MSL binding to 800 sites on X chromosome – 97% of them over genes and most near the 3' end, Kuroda said. MSL is attaching itself to H3K36me3, a targeting mark typically concentrated near the 3' end of genes and abundant on the X chromosome.

In another series of experiments, Kuroda set out to understand MSL's strong preference for binding the X chromosome and shunning autosomes. When the investigators inserted a *roX1* or a *roX2* genomic transgene in an autosome, MSL was eager to bind these familiar partners. They were surprised to find that on autosomes the MSL complex could also spread long distances from the original *roX* insertion site, attaching itself to active genes marked by H3K36me3 with a 3' bias.

Together, these data support a model in which MSL complex is initially restricted to a chromatin domain by high affinity sites such as *roX* genes, but then scans the chromosome for general marks such as H3K36me3. MSL complex and active chromatin marks such as H3K36me3 are conserved in mammals, Kuroda noted, improving the odds that this recruitment mechanism will prove important for more than *Drosophila.*

Histone Demethylases and Human Diseases

Yang Shi
Department of Pathology, Harvard Medical School

“Understanding biological importance is the next step,” Yang Shi said at the end of his 2006 symposium presentation. His lab pioneered the idea that histone methylation is dynamic when they cloned the first demethylase in 2004. Initially they could remove only one or two methyl groups from a lysine residue, and it seemed that trimethyl groups might be permanently attached. This was discouraging, because this type of histone modification appears to lock genes associated with various cancers and neurologic abnormalities in the “on” position.

Then, shortly before the 10th Armenise-Harvard Symposium last year, Shi’s team identified an enzyme family that knocked trimethyls off their histone perches in cultured human cells and live nematodes. In the nematodes, adding and removing methyl groups determined whether cells lived or died.

A hallmark of these proteins is the cruciform “Jumanji” domain, which is found in about 30 human protein families. The researchers screened these for demethylating enzymes and discovered four proteins that snipped groups or two or three methyls off medically interesting sites.

One of these is encoded by SMCX (or JARID1C), and certain point mutations in this gene are associated with X-linked mental retardation (XLMR). Although previous research forged links between these mutations and XLMR, exactly how these mutations damaged brain development was not known.

Shi now has an idea. SMCX lost demethylation capacity when point mutations found in XLMR were introduced into rat cerebellar granule neurons. Postsynaptic neurons no longer developed normal dendritic spines, an abnormality previously described in the brains of mentally retarded humans.

Separate experiments in zebrafish also raise the possibility that increased neuronal cell death may be partly to blame for XLMR. Researchers found that in zebrafish the homolog of SMCX, which acts exclusively in the brain during development, is essential for normal neuron survival.

Although there’s definitely more to learn, Shi notes that studies like these reinforce connections between basic chromatin biology and human disease.

Functional Interaction between the Nucleosome Remodeling Factor ISWI and Covalent Modifiers of Chromatin

Davide F.V. Corona
Istituto Telethon Dulbecco c/o Dipartimento di Scienze Biochimiche - Universita' degli Studi di Palermo

On TV makeover shows, swarms of carpenters, electricians and painters transform cramped tract houses into showplaces, shoving past one another to knock out walls and improve lighting.

The nucleus of a eukaryotic cell is not so different. Busy crews of epigenetic factors modify histones and remodel chromatin, pushing and pulling nucleosomes so that DNA is accessible, in a controlled manner, for transcription, repair, recombination, and other essential functions.

“All these activities integrate in the nucleus and they work at the same time,” Davide Corona said at the symposium, “but it’s not clear how their activities are coordinated to regulate chromatic structure, gene expression and other nuclear functions.” His lab uses genetic and biochemical tools to search for chromatin modifying factors that act in concert with ISWI, a highly conserved, ATP-dependent nucleosome remodeling factor.

In *Drosophila melanogaster*, ISWI regulates expression of about 5 percent of the genome and is involved in DNA replication, RNA transcription and chromosome organization, Corona said. Its activity can be modulated by site-specific acetylation of histones, and Corona’s team used flies with an inherited eye defect to screen hundreds of mutant genes that might be conspiring with ISWI to undermine normal chromatin condensation.

The screen identified two mutant genes of interest: SIN3A and PARP. Both are highly conserved enzymes involved in chromatin modification and dynamics.

SIN3A and its partner, RPD3, form a histone deacetylase complex involved in transcriptional repression. Immunostaining assays show that ISWI and this complex normally stick together on polytene chromosomes, but split apart on ISWI mutant chromosomes. A series of experiments confirmed interactions between ISWI and the SIN3A- RPD3 complex and revealed what Corona called “a functional antagonism between ISWI and the acetylation of histone H4 on lysine 16.” The researchers hypothesize that ISWI recruits the SIN3A-RPD3 complex to productively remodel nucleosomes.

The second interesting mutation was in the gene coding for the poly-ADP-ribose polymerase, or PARP, a chromatin enzyme that Corona said is important during DNA damage response and apoptosis. Heat shock proteins recruit PARP, which loosens chromatin by adding poly-ADP-ribose (PAR) and thus promotes transcription (which ISWI tends to repress). ISWI is poly-ADP-ribosilated *in vitro* and *in vivo,* which the investigators were surprised to find inhibits its ATPase activity by making it less likely to stick to the nucleosome. ISWI and PAR are localized in different chromatin domains on wild type polytene chromosome, but when ISWI is mutated PAR spreads on these chromosomes. Chromatin-bound ISWI can be jolted off polytene chromosomes by *in vivo* induction of chromatin poly ADP-ribosylation, Corona reported. This indicates that poly-ADP-ribosylation of ISWI inhibits its ATPase activity, causing ISWI to lose its grip on chromatin.

These data show different epigenetic factors working together as a network, with ISWI and covalent modifiers of chromatin regulating chromatin accessibility and other nuclear functions. Corona thanked the symposium organizers for the 2004 Armenise-Harvard Foundation Career Development Award, which made possible these findings and what he called “my new life in Palermo.”

Possible Roles in Silencing for piRNAs

Bob Kingston
Massachusetts General Hospital, Boston

Although many genes oscillate between talking and listening, some genes essential for maintaining particular tissue types must spend the life of the organism turned on in one cell type and off in another. Bob Kingston's lab is interested in epigenetic regulatory complexes that are as decisive as Tony Soprano: when they silence a gene, it stays that way. This silence is so profound that it is heritable, enshrined in chromatin and passed from one generation to the next.

In 2006, the Kingston lab and three other groups simultaneously described a new class of small regulatory RNAs that can render genes mute. These have been dubbed Piwi-interacting RNA, or piRNA, because they glom onto members of the Piwi family of proteins. These so-called Argonaute proteins interact with polycomb proteins, which since the 1940s have been known as heritable repressors of *Drosophila* genes.

Other laboratories working on piRNAs began with the protein-RNA complex, then figured out how to remove the RNA component for analysis. Knowing that these specialized RNAs occur in egg- and sperm-producing cells in fruit flies, members of the Kingston lab took a different approach.

Postdoctoral fellow Nelson Lau started with rat testes and set out to extract RNAs that might function as transcriptional gene silencers. He pulled out a complex containing small RNAs and Riwi, the rat version of human Piwi. The researchers dubbed this piRC.The small RNAs in piRC array themselves on chromosomes in a pattern typical for regulatory enzymes, Kingston noted. Approximately 94 percent of piRNAs map to 100 small (<100 kb) genomic loci; at these sites they adhere either to Watson or Crick strands, or if they bind both strands they do not overlap. These locations, Kingston said, appear to be conserved across species.

Preparations of piRC also hook up with rRecQ1, the rat equivalent of a helicase that helps silence genes in *Neurospora*. Recombinant RecQ1 and Piwi family proteins appear to interact directly, and in flies Piwi has been genetically linked to transcriptional gene silencing. Experiments show that the purified complex has piRNA-directed slicer activity, which adds weight to the idea that piRC is a heritable silencer of mammalian genes. Kingston said more evidence is needed to confirm this.

The Role of Chromosome 3D Organization in Polycomb-Dependent Epigenetic Regulation

Valerio Orlando
Dulbecco Telethon Institute at IGB CNR, Naples Italy

Profound shifts in gene expression enable cells to differentiate or change metabolic state. Much of the drama involves covalent modifications of DNA and chromatin and three-dimensional reorganization of chromosomes and genes in the nucleus. Together, these epigenetic factors collaborate to ensure the quality, stability, and heritability of cell-specific transcription programs.

Valerio Orlando wants to unravel the epigenomic mechanisms that control cell identity and cell fate plasticity. In particular, his lab studies Polycomb group proteins (PcG), which play a fundamental role in development, stem cell renewal and tumor progression by shaping chromatin structure.

In *Drosophila*, PcGs join with their target sequences, Polycomb Response Elements (PREs), to ensure epigenetic heritability of transcription programs and gene silencing. But how? One theory is that PREs are carpenters, building structures that sequester certain genes and make them inaccessible for transcription. Like houses, these structures may maintain the status quo by being passed from parent to offspring.

In a series of *in vivo* experiments with *Drosophila,* Orlando's team explored the dynamic, 3-D structure of the homeotic locus bithorax complex (BX-C).They used Chromosome Conformation Capture (3C) to identify where PcG binds to BX-C during early embryogenesis, then combined this information with data from fluorescent *in situ* hybridization (FISH) and FISH-Immunostaining (FISH-I) analysis.

They learned that when homeotic genes are repressed, PcG proteins, PREs and core promoters had interacted at a distance to build topologically complex structures. In contrast, when the homeotic genes are programmed to be active, PcG-controlled epigenetic DNA elements did not interact. Finally, Orlando reported that major changes in higher order structures are essential to maintain and stabilize alternative transcription states, while histone modification and reduced levels of PcG proteins define an epigenetic switch that is only partially heritable.

Future experiments will search for large and small RNAs involved in higher order structures within the nucleus, Orlando said, following clues that implicate the Dicer ribonuclease in changes to 3-D chromatin structures.

A Chromosome-Associated Small RNA Amplification Loop Required for Heterochromatin Formation

Danesh Moazed
Department of Cell Biology, Harvard Medical School

Heterochromatin was first described in 1928, when biologists realized that certain tightly compacted regions of chromatin stained differently from the rest. And only a few years later, *Drosophila* researchers recognized that genes are silenced in these regions, which are associated with centromeres, telomeres, repetitive DNA elements and cell differentiation genes, Danesh Moazed said in his opening remarks. One of the most fascinating properties of these domains is that they are epigenetically inherited even through many cell divisions.

Moazed has been interested in heterochromatin assembly and epigenetic inheritance for more than a decade, and initially he used budding yeast as a model system. More recently his lab began experimenting with *S. pombe,* or fission yeast, because its gene silencing mechanisms more closely resemble those of higher animals.

Three years ago, Moazed and his colleagues discovered surprising connections between RNA interference, or RNAi, and the assembly of heterochromatin at fission yeast centromeres – a process already known to involve histone binding proteins and methylation enzymes. They have since identified several multiprotein complexes that

physically link the RNAi pathway to heterochromatin assembly, and have analyzed their activities *in vitro* and *in vivo*.

As the Moazed lab fits pieces into this elaborate puzzle, it appears that a positive feedback loop deserves credit for keeping heterochromatin strong during cell division. A complex dubbed RITS attaches small RNA tags to specific chromosome regions that should be bundled into heterochromatin. Another complex, RDRC, works with RITS and the Dicer ribonuclease to generate a supply of small interfering RNA (siRNA). These siRNAs, together with histone H3 lysine 9 (H3K9) methylation, creates a positive feedback loop that generates more siRNA and maintains H3K9 methylation. The silencing mechanism involves co-transcriptional degradation of RNAs that are transcribed in heterochromatic domains by RNAi-dependent and -independent pathways, a process that Moazed calls CTGS, or Co-Transcriptional Gene Silencing.

The discovery of a direct physical connection between RNAi and heterochromatin raises the possibility of developing drugs that silence genes before they speak, rather than trying to counteract what they've already said.

Homologue Pairing: Regulating Genes and Driving Evolution

Ting Wu
Divisions of Genetics and Molecular Medicine, Harvard Medical School,

Ting Wu remembers a trip to Seattle, just a few years ago, where she first became interested in ultraconserved elements, or UCEs. These DNA sequences, at least 200 base pairs long, are virtually 100% identical in distantly related organisms. This struck a chord with Wu, whose lab has long been interested in homology and homologous pairing.

"Homology – or the number two – plays a huge role in the lives of cells," Wu said. She wondered if these UCEs, which are enriched in parts of the genome believed important for gene regulation and expression, might be involved in pairing. If so, they might also have something to do with copy number variations, situations where DNA segments do not march down the aisle in neat pairs. Her lab was already interested in odd numbers and the dynamic capacity of the genome to accommodate many deletions and duplications from one individual to the next.

Wu suspected there might be a connection between these two puzzles, and the report she heard in 2004 provided clues about what this could be. Researchers from the University of California - Santa Cruz had identified 481 unique UCEs conserved among humans, rats, and mice. These UCEs occur everywhere *except* chromosome 21 and the Y chromosome.

"What's intriguing about 21 and Y is they are the two chromosomes most likely to be found in humans in numbers other than two," Wu noted. "Trisomy 21 (Down syndrome) is the most viable human aneuploid and of course there is a single Y in males."

"We think that possibly these UCEs are likely to be copy-counters, and their role is to make sure that each segment of the genome occurs exactly twice," Wu said. She and her colleagues predicted that DNA segments present in copy numbers other than two would be depleted of UCEs.

Computational geneticist Adnan Derti helped test this model by determining whether three sets of UCEs, totaling 896 elements, are depleted among two sets of human segmental duplications (SDs) and seven sets of CNVs. They found a striking shortage of UCEs in the two sets of SDs ($P<10^{-8}$) and six of the seven sets of CNVs ($P<10^{-4}$). The results are consistent with "a stringent watching of copy numbers," Wu said.

Future experiments will look for specific motifs within UCEs and explore whether their duplication or deletion is associated with loss of fitness or death.

The Chicken-and-Egg Relationship between Transcription and Chromatin and Implications for Epigenetic Inheritance

Kevin Struhl
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School

Experiments with yeast have enabled Kevin Struhl and his colleagues to gain surprising insights into the relationship between the "beads on a string" structure for DNA storage and the apparatus that transcribes genes. They have learned that some transcription mechanisms do double duty: not only loosening heterochromatin into readable euchromatin but also repacking it in their wake.

In late 2005, Struhl and his colleagues made waves by demonstrating that RNA polymerase II (Pol II) does two seemingly opposite things at once. As it moves along the DNA template it not only exposes and reads genetic text, but also knocks off acetyl groups and covers up what it just read. Doing this keeps Pol II from mistakenly beginning a transcription partway along the coding region.

At the symposium, Struhl described how the epigenetic reprogramming of yeast illustrates the chicken-and-egg relationship between transcription and chromatin structure. The silencing protein Sir3 is an established tool for blocking DNA transcription, and it induces heterochromatin formation by causing rapid loss of histone acetylation. Removal of euchromatic histone methylation was more gradual, and over several cell generations the researchers observed structures that were neither euchromatic or heterochromatic.

Unexpectedly, Sir3 binding and the degree of transcriptional repression increased gradually for three to five cell generations, even though the intracellular level of Sir3 remained constant, Struhl reported. Strains lacking Sas2 histone acetylase or the histone methylases that modify lysines 4 (Set1) or 79 (Dot1) of H3 display accelerated Sir3 accumulation at *HMR* or its spreading away from the telomere, suggesting that these histone modifications exert distinct inhibitory effects on heterochromatin formation.

Methylation of H3K4 appears to represent what Struhl called a form of memory, a mark that says "I was recently transcribed." And this is the sort of mark that makes it possible for patterns of gene regulation to be epigenetically inherited. As for the relevance of these yeast studies to higher organisms, he cited the famous dictum from Jacques Monot: "What's true for *E.coli* is true for the elephant only more so."

Epigenome and Inflammation: Induction of a histone H3Lys27me3 demethylase by NF-kB – a link between inflammation and Polycomb-mediated gene silencing

Gioacchino Natoli
European Institute of Oncology (IEO), Campus IFOM-IEO, Milan

Inflammation is the immune system's first response to infection, irritation or damage, and its clinical manifestations range from the fleeting pain of a stubbed toe to chronic inflammatory disease or lethal septic shock. Gioacchino Natoli is intrigued by connections between inflammatory stimuli and epigenetic control of chromatin structure, especially the role of transcription factors in the NF-kB/Rel family.

In 2002, he and his colleagues reported dynamic changes in H3 Lys9 methylation in an experimental system that used lipopolysaccharide (LPS) stimulation of cultured human dendritic cells to simulate bacterial infection. Demethylation and remethylation of this site determined what sorts of inflammatory genes were transcribed and when.

More recently, Natoli and his colleagues have been experimenting with mouse proteins containing the "Jumanji" domain, murine equivalents of demethylating enzymes that Yang Shi's laboratory studies at HMS. The nuclear protein Jmj8 is induced by LPS stimulation only when NF-kB is present, Natoli said, and it removes the trimethyl group from H3Lys27me3 sites. When that group is attached, Polycomb repressive complexes – which silence genes – are induced.

The investigators next used microarrays to probe connections between H3Lys27 trimethylation and inflammation. Depleting Jmj8 did not change global levels of H3K27me3, but did increase basal levels at HoxA11, Natoli reported.

Additional experiments revealed that LPS stimulation had elicited dramatically different responses in different cell types. In myeloid precursors, the researchers witnessed sustained, high-level induction of Jmj8 and global control of HcLys27me3 levels. In differentiated macrophages, Jmj8 was transiently induced at a low level and there was no global impact on methylation, Natoli said.

This could mean that induction of this H3Lys27 demethylase by inflammatory stimuli generates a "permissive chromatin state" that allows one type of cell to turn into another. There have been signs of transdifferentiation in chronically inflamed cells of the eye, kidney, and intestinal lining, he said.

If future experiments confirm that transdifferentiation occurs during inflammation in his mouse models, due to epigenetic reprogramming, this could have many implications for human disease. Histone demethylases could turn out to be a direct molecular link between chronic inflammation and perturbation of differentiation programs (metaplasia) relevant for cancer development.

Growth Properties of Normal and Transformed Breast Stem Cells

Angelo Cicalese
Department of Experimental Oncology, European Institute of Oncology, Milan

Anti-cancer agents with epigenetic action are already being tested in patients with various lymphomas, and the ultimate goal of Angelo Cicalese's research is to identify epigenetic treatments for breast cancer. A recent series of experiments with mice suggests that this may be possible, Cicalese said at the symposium.

Like other tissues, tumors are organized hierarchically: they originate from and are maintained by a small subset of cancer stem cells, or CSCs. Cicalese and his colleagues asked whether epigenetic strategies could disable CSCs and shrink tumors.

To test this idea, they set up protocols for propagating breast stem cells from wild-type mouse mammary gland (BSCs) and transformed BSCs from MMTV-ErbB2 transgenic mice. BSCs not only survive in suspension, but also generate spherical cell aggregates called "mammospheres." Mammospheres are clonal in origin, show the stem cell properties of self-renewal and differentiation and are composed of BSCs and progenitors, Cicalese said. During serial passages, the number of mammospheres formed from wild-type mouse breast tissue decreased at a fixed rate through each passage. The ErbB2 mammospheres, in contrast, increased at an exponential rate and displayed a near-immortal phenotype. Mammosphere initiating cells, or MICs, accounted for the disparity; the growth rate of progenitors did not change over time.

To better study the growth properties of normal and transformed BSCs, the researchers incorporated a lipophilic dye, PKH26 (PKH), into stem cell plasma membranes. This allowed them to track a well-known property of stem cells: their ability to remain generally quiescent and/or undergo few divisions while giving rise to an intermediate population of actively proliferating progenitors.

Using this procedure with normal breast tissue, Cicalese's team found that only a minority of PKH+ cells continued forming mammospheres after being dissociated and replated several times. A few of the labeled cells were able to generate mammary tissue when transplanted into another animal's fat pad, showing that they are truly stem cells. BSCs from MMTV-Erb2 mice were an entirely different story. Both PKH+ and PKH- populations formed mammospheres *in vitro* and initiated tumors after transplantation, Cicalese reported. These ErbB2 mammospheres and breast cancer tissues contained more BSCs than normal mammospheres and tissue. The researchers attribute this to increased symmetric divisions of cancer BSCs, a type of growth pattern also seen in BSCs from mice missing the p53 tumor suppressor gene.

Knowing that mutated or functionally suppressed p53 is a common feature of ErbB2 tumors, the investigators speculated that p53 malfunction might account for increased symmetric divisions of transformed BSCs in their experiments. They used Nutlin3, an MDM2 antagonist, to block p53 degradation and restore its function. The result was a complete reversal of their earlier results, hinting that epigenetic treatments for breast cancer may be possible in the future.

Exit from Mitosis in Budding Yeast Saccharomyces cerevisiae

Rosella Visintin
Department of Experimental Oncology, European Institute of Oncology, Milan

Anyone who has ever beaten egg whites knows that timing is everything: whisk a few seconds too long and you'll have a mess, not a meringue. Mitosis is no different: cells must exit at precisely the right moment or run the risk of accumulating chromosome segregation errors and genetic instability associated with cancer.

Understanding how cells exit mitosis is the focus of Rosella Visintin's laboratory, which she founded in 2005 with support from a Career Development Award. She uses the budding yeast *Saccharomyces cerevisiae* as a model for studying cell cycle progression, which in all eukaryotes is triggered and coordinated by a set of proteins including cyclins, cyclin-dependent kinases (CDKs) and their inhibitors.

Phosphorylation of proteins by mitotic CDKs drives mitosis, and after chromosomes are accurately segregated these same CDKs must be inactivated so cells can leave mitosis and enter the G1 phase. In yeast, the phosphatase Cdc14 emerges from the nucleolus, where it has been bound to by the inhibitory subunit Cfi1, and spreads into the nucleus and cytoplasm. It zeroes in on its targets, brings mitosis to a sudden halt, and retreats swiftly to the nucleolus.

Visintin and her colleagues have been exploring how two regulatory networks, called FEAR (Cdc Fourteen Early Anaphase Release) and MEN (Mitotic Exit Network) control the association of Cdc14 with Cfi1 and promote Cdc14's release from the nucleolus. In her symposium presentation, Visintin focused on the flip side of the coin: mechanisms that rapidly inactivate Cdc14 after mitotic exit.

The protein kinase Cdc5, a component of both the FEAR and MEN networks, helps liberate Cdc14 from Cfi1 so it can leave the nucleolus. Work in the Visintin lab indicates that inactivation of Cdc5 is necessary – but probably not sufficient – to return Cdc14 to its storage place.

"Cdc14 plants the seeds for its own inactivation" via the FEAR and MEN networks, Visintin said, but exactly how this works remains to be seen. Answers from yeast will be relevant for humans, because the same players – Cdc14, FEAR and MEN – help regulate cell division and maintain genomic integrity in humans.

Dances-with-Retroviruses: The 40 Million Year Saga of the *TRIM5* Locus

Welkin Johnson
New England Primate Research Center, Department of Microbiology and Molecular Genetics, Harvard Medical School

Despite its global prominence for more than 20 years, in the long run the HIV/AIDS saga may be as fleeting as a CNN headline about the latest car bomb in Baghdad. The human genome is studded with the fossil footprints of retrovirus encounters stretching back tens of millions of years, Welkin Johnson said, long before AIDS. These battles between host and pathogen are memorialized in the germline and passed from one generation to the next as features of innate immunity.

One of these footprints is the TRIM5 locus. Since the early years of HIV/AIDS, researchers have realized that a genetic barrier kept HIV-1 from thriving in old world monkeys – making their cells unsuitable as models for HIV replication and AIDS. In contrast, human cells are catastrophically susceptible to HIV-1 infection, but able to repel some other retrovirus infections. A landmark 2004 article identified the rhesus monkey TRIM5 gene, and specifically the TRIM5□alpha splice-isoform, as a deal-breaker that blocks HIV replication in the cytoplasm, preventing the retrovirus from penetrating the host nucleus.

TRIM5α is a broad-spectrum antiviral whose specific capacities are shaped by each primate's history. When Johnson's team compared *TRIM5* from old and new world primates they found a high degree of interspecies divergence and evidence, from dN/dS comparisons, that strong positive selection was important. These observations motivated them to explore intraspecies diversity, or polymorphism, in the primate *TRIM5* locus. They began by surveying individuals representing three species, an African monkey (sooty mangabeys) and two Asian monkeys (rhesus macaques and pig-tail macaques). They were surprised to find a high degree of polymorphism, including multiple instances of shared polymorphism, Johnson reported. Both non-synonymous and synonymous SNPs were clustered in two regions of the gene, corresponding to two distinct domains in the TRIM5α□protein.

The human *TRIM5* locus, in contrast, was more monomorphic overall and displayed no variations in some regions that were highly polymorphic in other primates. For example, contemporary humans have an argenine allele at position aa334, where primates are polymorphic. The human allele is associated with susceptibility of cells to HIV replication, while primates with a proline in this position resist HIV. For millions of years the TRIM5α□ locus has been the scene of a struggle, Johnson speculated, between positive selection pressure and other forces determined to maintain multiple alleles at intermediate frequencies for millions of years, even as species diverged.

It is possible that only humans with the argenine allele survived a prehistoric retroviral epidemic, Johnson said, only to have the selection of that TRIM5 sequence open the door to AIDS millions of years later. If that is true, the TRIM5alpha story is biology's version of a Greek tragedy, where the hero's strength ultimately proves to be his undoing.

A Non-coding RNA Potentially Regulating 4q35 Genes Expression in FSHD

Davide Gabellini
Dulbecco Telethon Institute at Stem Cell Research Institute, DIBIT-HSR, Milan

The third most common form of muscular dystrophy is named for the bizarre way symptoms emerge, with patients gradually losing the use of muscles in the face, shoulder girdle and upper arms. Called facioscapulohumeral muscular dystrophy (FSHD), this autosomal dominant disease strikes approximately one in 20,000 people. The course of the illness varies, but the hallmark muscle groups waste gradually, other muscles weaken and many patients lose mobility with age. There are currently no treatments for FSHD.

The goal of Davide Gabellini's laboratory is to characterize the molecular pathogenesis of FSHD and develop new therapies. In 2002, he was part of a team that traced the disease to a deletion in the 4q35 region of chromosome four. This region contains the molecular equivalent of a study-hall monitor: it keeps bad boys quiet and in their seats, but remove it and they go wild.

The lost segment of 4q35 is not part of a protein-coding gene, but occurs in a non-coding repetitive element called D4Z4. Healthy people have 11 to 150 copies of this repeat; FSHD patients have one to 11 copies. D4Z4 is heavily methylated, has heterochromatic features, and contains a transcriptional silencer whose deletion permits inappropriate over-expression of nearby upstream genes.

Gabellini and his colleagues later identified three genes that became boisterous in cells with the FSHD deletion. One of them, dubbed FRG1 (for FSHD region gene 1) caused FSHD symptoms when over-expressed in transgenic mice, they reported in *Nature* in December 2006. The researchers also discovered aberrant alternative splicing in specific pre-mRNAs in the muscles of FRG1 transgenic mice and FSHD patients.

Additional studies in patient samples indicate that people with early-onset, severe FSHD have very few copies of D4Z4. Oddly enough, people devoid of D4Z4 are healthy and symptom free.

Although deleting D4Z4 is sufficient to create a mouse model for FSHD, Gabelleni says the loss of regulation of neighboring genes, such as FRG1, probably involves other factors as well. As for FRG1, it is probably one of a group of genes that affect RNA splicing, which in turn determines what proteins are made by cells. While his team continues to investigate the molecular pathogenesis of this fascinating disease, they also hope to use FRG1 transgenic mice to screen for possible therapies.