National Institute of Diabetes and Digestive and Kidney Diseases Symposium
Dynamic Epigenome and Homeostatic Regulations in Health and Disease

November 13 – 14, 2008
Bethesda, MD

NOVEMBER 13, 2008

INTRODUCTION

Olivier Blondel, Ph.D., National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Bethesda, MD

Interest in the epigenome and its involvement in disease development and progression has recently increased across the NIH. In 2008, NIH issued a series of initiatives to encourage research in epigenetic regulation, which has resulted in creation of reference epigenomes, new epigenomic markers, and other technological developments. Recently, NIH has fostered research related to the epigenomics of human health and disease.

At this time, it is too early to use epigenomic pathways and information to predict, prevent, and treat disease, but NIH nonetheless seeks to better understand the breadth of epigenomic regulatory processes and how these mechanisms participate in homeostatic regulation and explain interactions among biological processes, the environment, and disease development. Growth of this field will require cross-disciplinary collaboration along with support from NIH. Future RFAs likely will support epigenomic research, particularly under the Roadmap program.

This symposium included sessions organized around four broad themes: (1) Epigenetic Regulatory Mechanisms; (2) Aging, DNA Repair, Metabolism, and Inflammation; (3) Imprinting, Developmental Programming, and Transgenerational Inheritance of Epigenetic Marks; and (4) Of Environment, Sexes, and Food. Co-chairs for this meeting were Drs. Moshe Szyf, Michael Rosenfeld, Randy Jirtle, and Claudine Junien.

SESSION 1: EPIGENETIC REGULATORY MECHANISMS

Lessons Learned from Yeast about Human Leukemia

Ali Shilatifard, Ph.D., Stowers Institute for Medical Research, Kansas City, MO

Simple eukaryotes can be used to define highly conserved biological processes and the lessons learned from such studies can help answer questions about the genesis of complex diseases. Mixed lineage leukemia (MLL) is characterized by the presence of the MLL protein, a 4,000 amino acid protein that arises from a translocation of chromosome 11 to chromosome 4. At least 8 translocations are found in MLL cases, and these leukemias do not respond well to therapy.

Initial experiments to understand MLL function were undertaken in *Saccharomyces cerevisiae* because of difficulties in purifying the large MLL protein. The SET domain of Set1 in yeast is

highly homologous to MLL in humans and was used to isolate COMPASS – COMplex Proteins ASsociated with Set1. This complex contains Set1 as well as a number of other proteins, including the essential subunit, Cps35. COMPASS was the first histone H3 lysine (H3K4) methylase to be identified. Since then, a number of other SET-domain containing proteins have been characterized that are involved in methylation of lysine and arginine residues on histone tails. Human COMPASS contains SET1a, SET1b, ASH2, R55, WDR55, BPY30, and 4 MLL proteins and also has H3K4 methylation activity.

To identify other proteins that participate with COMPASS, global proteomic analysis of *S. cerevisia* was performed on extracts from 15,000 strains of yeast, each with a known gene knocked out. In this way, the pathway leading to COMPASS-mediated methylation was determined. Lysine 123 of histone H2B is monoubiquitinated by RAT631, which results in recruitment of the RNA polymerase II (RNA pol II) initiation complex. This complex serves as a platform for recruitment of COMPASS. A similar pathway functions in humans.

Analysis of COMPASS in the presence or absence of H2B ubiquitination showed that monoubiquitination of H2B requires H3K4 methylation. If COMPASS is purified away from wild type Rad6, or Rad6 is mutated such that it loses ubiquitination activity, or the ubiquitination sites in histone H3 are mutated, CPS35 cannot associate with COMPASS. The Paf1 protein complex, which associates with RNA polymerase II, is required for histone H3 methylation and recruits COMPASS to the chromatin. Rad6 activates the ubiquitination processes, which results in recruitment of CPS35 to the chromatin, where it activates COMPASS and methylation proceeds.

Human COMPASS contains MLL proteins and likely has a role in leukemogenesis. The human homolog of CPS35 is WDR82, which is a component of the human SET1 complex and is essential for H3K4 trimethylation. In cultured cells with WDR82 knocked out, a major reduction in H3K4 trimethylation is observed, but there is no effect on di- or monomethylation. In these cells, MLL 1-4 complexes are stable, but Set1 complexes are lost, indicating that MLL is not involved in all methylation, but that human COMPASS (or Set1) has an important role in global regulation of methylation. Set1 and MLL exist in a trimer with RbBP5, WDR5, and Ash2, which are required for methylase activity. Set1 is a more robust trimethylase than the MLL complexes, suggesting that the MLLs have specific roles in regulation of the pattern of H3K4 methylation. Knocking down WDR82, Set1a, or Set1b activity all result in reductions in global trimethylation. However, complete loss of MLL does not affect global trimethylation. MLL was found instead to mediate specific methylation at H3K4, which is an important marker for transcription activation.

MLL regulates H3K4 methylation of less than 5 percent of the genome. Many of the genes regulated by MLL are part of the Hox locus, which are important for development. MLL targets include Hox genes located in a region of the genome involved in hematopoiesis. MLL methylase activity could therefore induce aberrant activity of genes involved in hematopoiesis, leading to leukemia. Further analysis found 2,459 genes downregulated by loss of MLL; 299 of these are direct MLL targets. The yeast COMPASS complex was used to screen thousands of drugs for MLL inhibitory activity; compounds that inhibited COMPASS had nearly identical activity on

mammalian MLL. The similarities between yeast COMPASS and mammalian MLL permits drugs to be screened in yeast, verified on mammalian MLL in cultured cells, and then further tested in animal models.

The Dynamic Epigenome
Patrick McGowan, Ph.D., McGill University, Montreal, Quebec

The epigenome coordinates interactions between the environment and the inherited, static genome. Programming of the genome is controlled by the epigenome through modifications of chromatin structure and to the DNA itself. DNA methylation represents a stable signal that can be modified or perpetuated through development and in response to environmental signals.

DNA methylation patterns established during development are maintained by maintenance DNA methyl transferases (DNMTs) such as DNMT1. *De novo* DNMTs (DNMT3a, DNMT3b) that add methyl groups to previously unmethylated DNA also participate in the establishment of DNA methylation patterns. Other epigenome modifications include acetylation of the N-terminal tail of core histones, which leads to relaxation of the chromatin structure and increased access to the DNA. DNA methylation and histone acetylation appear to have inverse effects, and the two types of modification are spatially segregated in the nucleus. DNA methylation of regulatory elements can silence gene expression by inhibiting transcription factor binding at specific sites or by coordinating recruitment of other factors that impose a closed chromatin configuration, thereby blocking access to transcription factor binding sites.

The steady-state of DNA methylation is in dynamic equilibrium and is susceptible to influences from the environment, including physiological, social, toxicological, nutritional, and pathological stimuli. Evidence for dynamic DNA methylation also can be observed in post-mitotic tissue, such as the brain. Glucocorticoid receptor (*GR*) gene expression is influenced by the behavior of the mother to the offspring in rodents. Low levels of licking and grooming (LG) by the mother results in decreased expression of *GR* in the hippocampus of the offspring, leading to an enhanced stress response characterized by a poorer ability to attenuate the response to a stimulus. To determine the mechanism by which the effects of maternal care are maintained, the methylation pattern of the hippocampal $GR(1_7)$ promoter regions was analyzed in rats. In the embryo, mean methylation levels at this locus are low and rise considerably after birth. During the first week of life, pups receiving appropriate levels of LG from their mothers exhibit a decrease in methylation of this site; pups with low LG mothers maintained higher levels of methylation into adulthood.

The adult offspring of low LG mothers exhibit an enhanced stress response characterized by maintenance of high levels of corticosterone after exposure to stressful stimuli. Treatment of these pups with the histone deacetylation inhibitor trichostatin A (TSA) by injection into the lateral ventricles attenuates the corticosterone levels to the same degree as that observed in pups with high LG mothers. Blocking histone deacetylase action also results in hyperacetylation and an open chromatin structure, demethylation at the *GR* locus, and increased hippocampal *GR* expression in the pups with low LG mothers. The effects of maternal care on DNA methylation were reversed by administration of l-methionine, a precursor of the methyl donor S-adenosyl

methionine, which functions as a substrate for DNMT and therefore leads to increased methylation. L-methionine reverses the corticosterone response to stress in the adult offspring of high LG mothers; these animals now exhibit behavior similar to the offspring of low LG mothers. This work has shown that the environment can introduce epigenetic changes that re-program gene expression and result in distinct phenotypes.

To determine if similar mechanisms function in humans, alterations in gene expression patterns in the brains of suicide victims who were severely abused in childhood were examined. In this analysis, methylation at the rRNA promoter, which regulates its transcription, was examined. The genome contains more than 400 copies of rRNA genes and these genes represent a bottleneck for protein synthesis. Hippocampal volume and neurogenesis are decreased in major depressive disorder and transcription and protein synthesis are decreased in early stages of Alzheimer disease. Reduced rRNA gene expression and rRNA hypermethylation was observed in the brains of the suicide subjects. No informative SNPs were identified in the rRNA promoter among the suicide subjects and controls. Analysis of the cerebellum found no differences in methylation between suicide and control subjects. Analysis of 38 CpG sites in the brains of 12 childhood abuse and suicide victims, 12 non-abused suicide victims, and 12 controls found increased methylation of the *GR1f* promoter region and decreased *GR* expression in the hippocampus of abused suicide victims compared to non-abused subjects and controls.

This work has demonstrated that environmental (i.e., chemical and social) stimuli can induce epigenetic changes that, together with inter-individual variation, alter gene expression and signaling pathways. Changes in the epigenome can result in altered gene expression programming and altered responses to the environmental stimuli, resulting in detectably different phenotypes.

Structural Biology of Epigenetic Regulation: How Chromatin-Binding Modules Interpret Histone Modifications

Dinshaw J. Patel, Ph.D., Memorial Sloan-Kettering Cancer Center, New York, NY

The bromodomain PHD finger transcription factor (BPTF) is the largest subunit of the chromatin remodeling complex and couples H3K4 trimethylation with chromatin remodeling. The PHD2 finger of BPTF serves as the methylation reader module of this complex and the bromodomain serves as the acetyl-lysine reader. These two domains are precisely separated by an □-helical linker region. The PHD2 finger has an aromatic surface cage that recognizes methylated lysines. Analysis of the structure of this complex revealed a structural basis for its specificity. The trimethyl lysine residue at position 4 of the histone H3 N-terminus inserts into the aromatic cage of the PHD2 region. An arginine residue on H3K4 also helps to position the histone tail in the cage; the recognition motif for this interaction is arginine-X-K4me3. Very little conformational change is observed in the structure of the PHD2 finger as it transitions from a free to bound state.

Mutating specific residues in the aromatic cage changes the specificity for the methylation state of K4. The wild type protein binds trimethylated lysines better than dimethylated, and binds dimethylated better than mono-methylated. Replacing tyrosine 17 with an acidic amino acid alters specificity such that the dimethylated lysine binds with higher affinity. The acetylated

lysine residue at position 16 of histone H4 is recognized by and inserts into the pocket of the bromodomain. The combination of PHD finger and bromodomain, separated by a fixed distance, results in a dual reader that recognizes two distinct marks on the histone. This reader module can recognize peptides within the tails of the histone as they project off the nucleosome, resulting in correct orientation of the PHD-bromodomain complex.

MLL is a large protein with a methyltransferase SET domain and other modules, including a PHD-bromodomain. Analysis of the structure of this protein found a PHD finger and bromodomain separated by a 6 amino acid linker that is partly □-helical and extends into the □-helix of the bromodomain. In MLL, interactions between the PHD finger and bromodomain lock this protein into a large globular architecture. Superimposing MLL and BPTF showed that the positioning of the bromodomains in these proteins is different and thus MLL and BPTF interact with the nucleosome in different orientations.

MLL bound tri- or dimethylated K4 with relatively high affinity; binding to monomethylated K4 was at least 10-fold weaker. Mutating amino acids surrounding the aromatic cage of MLL abrogates binding. The MLL1-PHD3 finger complex undergoes conformational change and forms an aromatic cage dependent on the methylation state of the peptides, indicating that the MLL pocket is not pre-formed as is the case for BPTF. The N-terminus of the histone also is more accessible in the BPTF complex and is covered by MLL interaction. Extending the N-terminus of histone H3 by adding alanine had a small affect on BPTF binding; binding was further reduced when 3 alanines were added. Extension of the N-terminus had a more pronounced affect on MLL binding, which was lost completely upon addition of 3 alanines, because the N-terminus is buried further within MLL than BPTF.

JARID1A is a histone demethylase with jumonji domains and 3 PHD fingers. JARID1A is often found attached to a common translocation partner, nuclear protein 98 (NUP98), through PHD3. JARID1A+NUP98 is an oncoprotein that arrests hematopoietic terminal differentiation and induces acute myeloid leukemia. PHD3 recognition of trimethylated H3K4 is essential for leukemogenesis; mutations in PHD3 that abolish H3K4 binding also abolish leukemogensis. Fusion of these proteins also induces persistent transcription of HoxA5-A10 loci, locking this region in an active epigenetic state by promoting inappropriate acetylation and preventing silencing of the locus. Alignment of JARID1A PHD fingers with MLL and BPTF showed that JARID1A lacks some aromatic amino acids found in the other two proteins. JARID1A binds dimethylated and trimethylated lysines equivalently, binds monomethylated residues weakly, and does not bind unmethylated residues. Two tryptophan residues are required for formation of the aromatic cage, and the lysine channels within this cage are separated by distances different from those found in MLL and BPTF.

The malignant brain tumor (MBT) repeat is a protein module structurally similar to domains that bind to methylated histones and causes malignant transformation in Drosophila brain. H3L3MBTL1 is a transcriptional repressor that compacts nucleosomal arrays dependent on post-translational methylation modifications. MBT domains have a triangular architecture; the N and C termini are located close to one another and each MBT repeat has a repeated motif of extended and globular segments. One face of the triangle has pockets on each of the three globular

domains, corresponding to each MBT repeat. MBT domains bind mono- and dimethylated H3K9, but not trimethylated. Pocket 2 of the MBT domain binds the methylated lysine; the pocket is too narrow to bind trimethylated lysine. A proline residue at position 30 on histone H3 is recognized by pocket 1, along with methylated lysines at positions 36 and 37. In contrast to other PHD-containing proteins, MBTL1 recognizes histone modifications in its pockets rather than via a surface channel. The dimensions of the pocket thus are important for appropriate recognition.

Computational Methods for Genome-Wide Prediction of Imprinted Genes
Alexander J. Hartemink, Ph.D., Duke University, Durham, NC

Imprinted or epigenetically modified genes play important roles in embryonic development; development of conditions such as cancer, obesity, and autism; and in response to environmental exposures. Imprinting at specific loci in response to environmental factors also can lead to stable and heritable changes in phenotype. For example, methyl supplementation of pregnant agouti mice can lead to methylation at the agouti locus; offspring carrying this epigenetic mark are obese, yellow, and more likely to develop tumors compared to genetically identical mice in which the locus is not methylated.

Developing an algorithm to predict imprinting is difficult, because the inputs from complex biological systems are noisy, imperfect, and often of different types generated by different assays. The desired output of a predictive algorithm is a model that provides good explanatory or predictive power for recognizing patterns in the input data. These algorithms must be able to integrate evidence from multiple sources of noisy data for efficient probabilistic learning. The goal of such a predictive model for imprinted genes is to identify novel imprinted genes and determine how they arose and whether they are linked to a disease process.

A machine learning approach using a sparse multinomial logistic regression software package for identifying imprinted gene candidates and predicting parental expression preferences was developed by training classifiers using known imprinted and non-imprinted genes and a series of DNA sequence features such as CG content, transcription factor binding sites, and the presence of retrotransposons all located within approximately 100 kb of the gene. Training cut the initial list of approximately 7,000 features to approximately 700. These classifiers were applied to the whole mouse genome to identify potentially imprinted genes; genes with human orthologs in regions known to exhibit parent-of-origin inheritance were highlighted. The refined classifier set also was applied directly to the entire human genome. Comparison of putative mouse and human imprinted genes demonstrated that the overall prevalence of imprinting in humans was approximately half that observed in the mouse genome. Maternally-expressed genes also were more common in the mouse genome. Of the 119 putatively imprinted human genes with a mouse ortholog, only 32 percent also are imprinted in mice. These differences have significant implications for the use of mice as models of human diseases.

Experimental validation of putative imprinted genes is extremely difficult. Monoallelic expression may occur in only a specific cell type or at only a certain stage of development (usually early in development). Monoallelic expression must be confirmed in many individuals

to ensure parent-of-origin dependent expression, rather than random silencing. In addition, the large set of selected features for imprinted genes is difficult to interpret. At present, five new imprinted human genes have been experimentally validated. Two of these genes are located on chromosome 8, which was not previously known to contain any imprinted genes. The prediction tool itself is agnostic and does not recognize species; if two genomic regions are identical, the same predictions will be made. Development of a "universal imprint predictor" is possible if the imprinting mechanisms for establishing and reading epigenomic marks are the same across species.

Epigenetic Regulations by Molecular Clocks
Joe Bass, M.D., Ph.D., Northwestern University, Evanston, IL

The mammalian circadian system organizes many physiological and metabolic processes in synchrony with the 24-hour rotation of the earth. The core clock is located in the master pacemaker neurons of the suprachiasmatic nucleus (SCN) but the entire clock network includes extra-SCN regions of the brain and peripheral tissues. Evidence suggests that circadian regulation is linked to metabolic homeostasis and dysregulation of the circadian system contributes to obesity and diabetes.

To identify mice mutated in circadian rhythm, patterns of activity were analyzed; mice characteristically are more active at night. An animal with a period of activity exhibiting a 6-fold standard deviation from normal was identified and found to have a point mutation in the *clock* gene. The mutation behaved in an autosomal dominant manner and was found to function as a dimer with its partner, BMAL1 by binding at an E-box motif. The clock-BMAL1 dimer regulates expression of *mPer1*, *mPer2*, and *mPer3*, which together with *mCry1* and *mCry2* form a complex that inhibits the clock-BMAL1 dimer. Clock activity regulates metabolic target genes both in the central nervous system (i.e., neuropeptides in the hypothalamus) and peripheral targets (genes involved in glucose and lipid metabolism). Levels of liver proteins such as the hepatic nuclear factors (HNFs), and proteins involved in gluconeogenesis oscillate in response to clock-BMAL1 activity at the E-box. Nuclear hormone receptors exhibit similar rhythmic light-dark oscillations.

Feeding behavior is synchronized with the sleep-wake cycles. In the lateral hypothalamus, orexin is regulated by wakefulness and in turn regulates feeding. Ablation of the orexin receptor causes anorexia and alterations in periods of wakefulness in mice, providing evidence for the coordination of eating and waking patterns. The *clock* mutant mouse also exhibits attenuation of activity at the onset of a dark cycle, in contrast to wild type mice that wake and eat during darkness periods. To test if *clock* mice also have defects in metabolism, these mice were fed a high fat chow and their weight gain compared to wild type mice fed the same diet. The *clock* mutant mice gained more weight than the wild type mice and had a 25 percent increase in adiposity. The *clock* mutant mice also increased their energy intake and did not restrict their eating to dark cycles. Although food intake for both mutant and wild type mice was dysregulated in the presence of the high fat diet, the phenotype was exaggerated in the *clock* mutants. The *clock* mutant had abnormal glucose metabolism and fat storage patterns, and appeared to develop metabolic syndrome, characterized by hyperglycemia, hyperlipidemia, and

hyperleptinemia. These data demonstrate the interaction between the defect in *clock* and the inability to control body weight when fed a high fat diet and suggests that *clock* has a global effect on metabolic homeostasis.

Clock regulates hypothalamic transcript expression rhythms and abundance. Orexin and ghrelin, which are expressed during the feeding activity cycle in mice, appear to be regulated by *clock*. Expression of *clock* itself can be altered by a high fat diet. Mice fed a high fat diet exhibit a gradual lengthening of periods of activity and rest, implying that clock activity may be genetically programmed but also is sensitive to diet. Mice fed a high fat diet began to eat approximately 30 percent of their food during what is normally the rest period and the extra calories consumed by these animals were consumed at the wrong time during the circadian cycle. Analysis of gene expression in fat and the liver showed that expression of *clock*, *Bmal1*, and *Per2* are altered by the high fat diet.

Recent evidence suggests that rhythmic NAD+ biosynthesis is involved in regulation of the clock network. NAD+:NADH balance affects transcription of NPAS2, a clock protein expressed in the forebrain, with NADH promoting DNA binding by a NPAS2-BMAL dimer. NAD+:NADH balance also regulates Silent Information Regulator 2 (SIR2), which is involved in the longevity response to caloric restriction. SIR2 is an NAD+ dependent protein deacetylase that deacetylates histones, resulting in chromatin compaction and loss of access for transcription factors. NAMPT (PBEF/visfatin), which controls NAD+ biosynthesis, normally oscillates in liver, but this oscillation is lost in the *clock* mutant. NAD levels also increase during active periods and are significantly decreased in *clock* mutants, demonstrating that NAD levels are regulated in a circadian manner. Clock itself is a histone acetyltransferase (HAT) that catalyzes acetylation of lysine residues on histone tails, thus opening condensed chromatin. Acetylated histone 3 is associated with transcriptionally active *Per1*, *Per2*, and *Cry1*. A yeast 2-hybrid screen found that SIRT1, the mammalian homolog of SIR2, interacts with BMAL inhibits the BMAL-clock dimer. *Per2* expression increases in the presence of Sirt1 inhibitors such as nicotinamide and resveratrol, demonstrating that SIRT1 is a *Per2* inhibitor. SIRT1 and NAMPT thus represent key coordinators of the metabolic and circadian pathways. Interactions between these pathways control metabolic processes such as gluconeogenesis, lipolysis, and glucose-stimulated insulin secretion and thus may in part explain the contribution of circadian dysregulation to conditions such as obesity and diabetes.

SESSION 2: AGING, DNA REPAIR, METABOLISM, AND INFLAMMATION

Life, Death, and Transformation: Movements, Repeats, and ncRNAs

Michael G. Rosenfeld, M.D., University of California at San Diego, La Jolla, CA

Genetic Variation in Genome Maintenance and Aging

Yousin Suh, Ph.D., Albert Einstein College of Medicine, Bronx, NY

Aging is a major risk factor for the most common human diseases and one of the most complex phenotypes that we know. The identification of genetic variation and their potential functional

impact on aging-related phenotypes will be important in assessing genetic components of aging, including exceptionally healthy aging, ultimately contributing to our understanding of the functional diversity in aging human populations. We hypothesize that genetic variation at loci involved in genome maintenance can be related to individual differences in the rate and severity of aging. We are conducting a systematic multidisciplinary study to discover "functional gene SNP haplotypes", i.e., allelic variation caused by multiple SNPs in the same gene, among over a hundred candidate genes acting in genome maintenance pathways. These candidate genes include all genes in which heritable mutations have been found to be associated with accelerated aging in humans or mice, as well as genes interacting with these key genes and other genes acting in the same pathway. To ascertain the functional relevance of observed positive associations, candidate gene-SNP haplotypes are screened for various parameters of cellular fitness in short-term cell culture studies. Functionally relevant gene variants will then be further studied for their in vivo effect during aging by modeling them in the mouse. We will present an example of gene variants related to a major aging-related disease.

DNA Damage Responsive Pathways

Junjie Chen, Ph.D., Yale University School of Medicine, New Haven, CT

The breast cancer susceptibility gene 1 (BRCA1) plays an important role in the DNA damage response. Mutation of BRCA1 leads to increased sensitivity to DNA damage and genomic instability, and BRCA1 mutations are implicated in nearly 80 percent of familial breast cancer cases. The role of BRCA1 in DNA damage response and repair was suspected when BRCA1 protein was found to localize to foci of DNA damage. BRCA1 is a 1,863 amino acid protein with an N-terminal zinc finger motif with ubiquitin ligase activity and C terminal BRCT (BRCA1 C terminal) motifs that bind protein.

The initial event occurring in the cell's response to a DNA double strand break is activation of the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and rad3-related (ATR) protein kinases. The exact mechanism by which the break is initially sensed is not clear, but may involve binding of a complex that includes MRE11, RAD50, and NBS1, which in turn activate ATM and ATR. ATM and ATR activate downstream checkpoint kinases; BRCA1 may link the sensing of double strand breaks to activation of these cell cycle kinases.

Phosphorylation of H2AX, a unique isoform of histone H2A, by ATM, ATR and others is the initial event occurring after a DNA break occurs. Phosphorylated H2AX is required for recruitment of other kinases, mediators, and DNA repair proteins to the site of the break. Through its BRCT domain, mediator of DNA damage checkpoint protein 1 (MDC1) is recruited to the site of the break by phosphorylated H2AX. MDC1 in turn recruits ATM, which leads to further phosphorylation of H2AX and increased recruitment of MDC1, amplifying the break signal. Amplification thus creates a large pool of MDC1 at the site of the break.

After amplification and MDC1 recruitment, checkpoint and repair proteins including BRCA1 and the MRE11, RAD50, and NBS1 complex are recruited. Recruitment of this complex depends on interactions between MDC1 and NBS1 and is regulated by CK2-mediated phosphorylation of conserved sites on MDC1. This phosphorylation is constitutive and

independent of DNA breaks. Recruitment of BRCA1 to break sites is initiated by interactions between MDC1 and H2AX, which recruits ATM to the site. MDC1 and ATM interact with E3 ubiquitin ligase. Together with UBC13, E3 ligase promotes formation of ubiquitin chains near the site of the breaks and these chains recruit BRCA1. BRCA1 does not directly recognize the ubiquitin chains; recruitment is mediated by the CCDC98 (abraxa) complex and Rap80. UBC13 promotes formation of K63-linked ubiquitin chains, which bind RAP80 and recruit other proteins involved in DNA repair, such as PTIP, 53BPI, and RAD18. RAD18 binding to ubiquitin chains is dependent on its zinc finger ubiquitin binding motif. RAD18 recruits RAD51C and RAD51, which are essential for homologous recombination.

BRCA1 is most significantly involved in homologous recombination repair through its interactions with BRCA2 and the RAD51 complex. The structures of BRCA1 and BRCA2 are unrelated, but the two proteins have been found to stably interact in mitotic and meiotic cells; BRCA1 also interacts with RAD51 in these cells. BRCA2 has BRC repeats that bind RAD51 and also has a DNA binding domain, thus enabling BRCA2 to recruit RAD51 to DNA to promote homologous recombination. BRCA1 and BRCA2 interact through the pre-BRCT domain on BRCA1, but this interaction is most likely mediated by the linker protein Partner and Localizer of BRCA2 (PALB2). PALB2 is involved in homologous recombination repair of double strand breaks and is mutated in some familial breast cancer patients and in Fanconi anemia. A coiled-coil domain on PALB2 is required for interaction with the pre-BRCT domain of BRCA1, while WD40 repeats on PALB2 interact with BRCA2. PALB2 interaction with BRCA1 is required for efficient homologous recombination repair through its role in facilitating recruitment of BRCA1 to the DNA break site.

The Epigenome in Inflammatory and Antimicrobial Transcriptional Responses
Gioacchino Natoli, M.D., European Institute of Oncology, Milan, Italy

The antimicrobial response and both normal and pathological inflammation are characterized by coordinated changes in the expression of several hundred genes, to ensure that the correct gene is expressed in the correct location with the correct kinetics. Many of these genes are direct targets of the NF□B/Rel family of transcription factors. Inflammation is the outcome of the innate immune response, although a similar response can be induced by non-microbial damage. Recognition of microbes by cell surface receptors is followed by nuclear integration of signals induced by microbe recognition, leading to transcription. Hundreds of genes are differentially expressed in response to a stimuli and this complex response relies on a small number of transcription factors, such as NF□B.

The kinetics of NF□B recruitment and transcriptional induction in response to a stimulus defines two classes of inflammatory genes. The first consists of constitutively active and immediately accessible genes, which are mainly involved in the innate immune response. These genes are characterized by high CG content in the promoter; constitutive H3K4 trimethylation and H3/H4 acetylation; no requirement for Swi/Snf chromatin remodeling complexes; and immediate recruitment of NF□B/Rel transcription factors. The second group includes genes with regulated and late accessibility, including several involved in T cell recruitment and activation. The promoters of these genes have relatively low CG content; low to undetectable H3K4

trimethylation and histone acetylation in unstimulated cells, followed by a progressive increase in H3K4 trimethylation and H3/H4 acetylation after stimulation; a requirement for Swi/Snf chromatin remodelers for NF□B recruitment, H3K4 trimethylation, and gene activation; and, in some cases, a requirement for the coactivator I□B□, specifically for H3K4 trimethylation.

Searching for genes induced by inflammatory stimuli found that the JmjC protein, Jmjd3, is undetectable until it is induced in activated macrophages by LPS + □IFN. The Jmjd3 locus has one of the highest contents of conserved NF□B binding sites, second only to I□B□, and also has a number of H3K4me3 peaks. Jmjd3 itself is an H3K27me3 demethylase and is incorporated into H3K4 methyltransferase (MLL) complexes. Several genes associated with H3K27 trimethylation encode master controllers of developmental programs or proteins restricted to specific differentiated cell types. The H3K27 mark is transmissible across mitosis and mediates the silencing of many genes, including the master controllers of cell fate and development. Thus, this mark can prevent lineage-inappropriate gene expression.

Inflammation also induces H3K27 demethylation, suggesting that Jmjd3 could be a link between inflammation and epigenetic alterations that lead to abnormal tissue differentiation. For example, induction of an H3K27me3 demethylase by inflammatory stimuli may explain the link between inflammation and alterations in tissue differentiation (e.g., metaplasia in chronic gastritis). However, this apparent link does not explain the evolutionary basis for selection of a strong association between Jmjd3 and an inflammatory transcription factor such as NF□B. To explore this relationship, analysis of the genome-wide distribution of Jmjd3 was performed, along with creation and analysis of Jmjd3 knockout mice. Chromatin immunoprecipitation (ChIP) analysis found 3,339 genes with evidence of Jmjd3 binding within a 1 mBP region of chromosome 5. Binding was nearly always associated with transcription start sites. Jmjd3 target genes are involved in leukocyte migration and activation, microbe recognition and killing, and inflammatory gene transcription, essentially every process needed for an immune response.

In activated macrophages, Jmjd3 was recruited to transcriptionally active genes with high H3K4me3 content and RNA polymerase II (RNA pol II) occupancy; 73 percent of Jmjd3 target genes showed increased Pol II occupancy after LPS stimulation. Most (88%) Jmjd3 target genes had low to undetectable levels of H3K27me3 at their start sites before LPS stimulation. Analysis of changes in H3K27me3 induced by LPS treatment of macrophages showed a greater than 2-fold reduction in 16.3 percent of H3K27me3 peaks associated with Jmjd3 ; the strong reduction in H3K27me3 signals at Jmjd3 target genes reflected nucleosome loss rather than demethylation.

Analysis of fetal liver macrophages from a *Jmjd3* -/- knockout mouse found that RNA pol II binds in the absence of Jmjd3, but Jmjd3 is required to sustain RNA pol II occupancy. Downregulation of Jmjd3 target genes also is observed in these macrophages. The transcriptional effects of Jmjd3 absence appear to be independent of K27me3 demethylation. Taken together, these data demonstrate that Jmjd3 is preferentially associated with the start sites of hundreds of active and LPS-inducible genes and is required to sustain Pol II occupancy at these genes. Thus, Jmjd3 is the effector of an NF□B-triggered feed-forward transcriptional loop whose function is to sustain transcription of inflammatory and anti-microbial genes.

Redistribution of SIRT1 as a Cause of Age-Related Changes in Genomic Stability and Gene Expression

David A. Sinclair, Ph.D., Harvard Medical School, Boston, MA

The mechanisms that regulate changes in gene expression that occur during aging are generally unknown. Redistribution of chromatin factors is observed in yeast as they age, implying a role for epigenetic changes in aging. Areas of heterochromatin repress transcription, ensuring that genes are expressed appropriately with respect to time and location. As aging progresses, heterochromatin accumulates and suppresses transcription of genes associated with youth, while heterochromatin is lost and inappropriate transcription of other genes increases. These age-associated gene expression changes share similarities with changes in expression associated with oxidative stress.

Yeast have been used as a model system to explore aging. Ribosomal DNA in yeast is repetitive, highly unstable, and contains approximately 200 repeats that can recombine readily and form extrachromosomal rDNA circles (ERCs). Accumulation of ERCs contributes to yeast aging. The Silent Information Regulator genes, of which *Sir2* is the best known, silence mating type genes and help maintain genomic stability. Once the silent mating type genes are activated, the yeast become sterile, which is the key phenotype of yeast aging. Introducing two copies of *Sir2* into yeast slows the creation of ERCs and increases lifespan by approximately 30 percent.

The redistribution of SIR2 protein in response to genome instability underlies the loss of youthful gene expression patterns. During the aging process, SIR2 protein is recruited to the rDNA locus in the nucleolus by induction of a DNA break. The break recruits the SIR2 complex and activates silent genes, in a checkpoint-dependent process that requires MEC1 (the yeast version of ATM kinase). The mating type loci also are de-repressed by oxidative stress, which is associated with aging. Addition of extra copies of *Sir2* counteracts de-repression caused by peroxide, reduces recombination frequency, thus moderating genome instability, and preventing sterility in yeast. These effects translate to an increased lifespan for yeast.

Mammalian Sir2 (SIRT1) also is recruited to areas of DNA damage. In the presence of peroxide or alkylating agents, SIRT1 is more tightly associated with chromatin. The ATM response also is required for this association, and SIRT1 recruitment is improved in the presence of H2AX. In mammalian cells, SIRT1 appears to be essential for chromosome stability in the presence of stressors such as oxidative agents, but not for stability under normal conditions. An assay for DNA repair in mammalian cells was used to analyze the efficiency of repair and determine if SIRT1 is recruited to the break site. In cell lines in which SIRT1 levels were knocked down by 50 percent, a loss of DNA repair efficiency by homologous recombination was observed; NHEJ repair also was affected, but not as severely. ChIP analysis found SIRT1 at DNA breaks in normal cells, but not in the knockdown cells. SIRT1 also was required for recruitment of DNA repair factors RAD51 and NBS1. Increasing SIRT1 levels in *p53* +/- mice protects these mice against radiation-induced lymphoma and delays loss of heterozygosity, indicating improved efficiency of DNA repair.

To explore whether SIRT1 relocalization across the genome could promote the changes in gene expression that occur during aging, genome-wide promoter distribution of SIRT1 and histone H1-AcK26 (a marker of SIRT1 activity) were analyzed. An inverse correlation between SIRT1 binding and H1-AcK26 was observed. Approximately 400 genes that bind SIRT1 were identified in mice, including genes involved in metabolism, oxidative stress response, chromatin assembly, DNA packaging, aging, and nucleotide excision repair. After inducing DNA damage with peroxide, loss of SIRT1 at these sites was observed, suggesting that SIRT1 moves to stochastic sites of DNA repair. To determine whether these mechanisms resemble aging *in vivo*, SIRT1 binding was compared in brains from 5-month old mice versus 30-month old mice. This analysis found changes in transcription patterns of SIRT1-regulated genes. Over-expression of SIRT1 in brain was hypothesized to prevent these transcriptional changes. In a SIRT1 transgenic mouse (NeSTO), brain-specific SIRT1 over-expression prevented the age-associated changes in gene expression normally observed. However, the benefits of this are unknown, as inappropriate over-expression of some genes in neurons could lead to aberrant apoptosis.

O-GlcNAc: A Novel Epigenetic Mark Deregulated in Metabolic Syndrome
John Hanover, Ph.D., NIDDK, Bethesda, MD

O-linked β-N-acetylglucosamine (O-GlcNAc)-modified proteins include transcription factors, nuclear pores, proteasomal subunits, and signaling kinases. O-GlcNAc is derived from nutrients and integrates nutritional information and the hexosamine signaling pathway that leads to the reversible addition of O-GlcNAc to target proteins is involved in cellular response to nutrient excess. O-GlcNAc modifications also impact the IGF signaling axis and thus insulin resistance and obesity.

O-GlcNAc transferase (OGT) adds O-GlcNAc residues to proteins and is opposed by O-GlcNAcase (OGA), which removes O-GlcNAc residues; both OGT and OGA activity are modified by nutrient input. OGT is a highly conserved and essential protein, hypothesized to have a role in the development of diabetes. OGT transcripts are highly enriched in pancreatic cells and OGT itself is derived from the hexosamine biosynthetic pathway. The OGA locus is associated with susceptibility to diabetes in Mexican Americans, and to a lesser extent in Pima Indians. Complete knockout of OGT in mice is lethal during embryogenesis, but other OGT-modified transgenic mice developed insulin resistance.

Over-expression of OGT in muscle and adipose tissue in mice results in insulin resistance and hyperleptinemia. Modification and activation of endothelial nitric oxide synthase (eNOS) by OGT is associated with erectile dysfunction in men with diabetes. O-GlcNAc modification of CRCT2 increases hepatic glucose production in diabetes. OGA occurs in two difference forms, one of which associate with chromatin and the other with lipid droplets. OGA levels are dictated by the levels of neutral lipid droplets, demonstrating another route by which O-GlcNAc can act as a nutrient sensor. Advanced glycation end products recruit OGT to the chromatin, and both OGT and OGA may be important for modifying histones. Along with GlcNAc removal activity, OGA also has a histone acetyltransferase (HAT) domain and binds histone H3 tails.

C. elegans has been used to model type 2 diabetes and explore the roles of OGT and OGA in this condition. In *C. elegans*, ogt1 and oga1 modulate insulin signaling. A temperature sensitive allele of the insulin receptor daf2 was used to induce a developmental switch called the dauer larval stage, an alternative life form adopted by *C. elegans* when nutrients are scarce. Crossing the daf2 allele to an oga1 knockout increased dauer formation; crossing to an ogt1 knockout reduced dauer formation. Analysis of gene expression in the ogt1 knockouts found highly increased transcription of c type lectins, insulin peptides, and genes involved in stress and anti-microbial response. These effects correspond to insulin resistance and insulin hypersensitivity, suggesting that ogt1 suppresses insulin signaling. This system also was used to analyze transcriptional regulation by O-GlcNAc; between 650 and 700 genes out of approximately 20,000 in the *C. elegans* genome carry the O-GlcNAc mark. The genes were involved in nutrient-sensing pathways and preferentially marked at promoter regions, particularly for genes with multiple transcriptional start sites. O-GlcNAc usually marks promoters upstream of the first gene in an operon. Maternal exposure to increased glucose appears to increase O-GlcNAC marks in the offspring.

Studies in *C. elegans* and mice suggest that OGT and OGA are involved in nutrient response and may have roles in the development of diabetes and obesity, as well as in the innate immune response. In mice, knocking out OGT resulted in thymocyte apoptosis; OGA knockouts showed 3-fold higher expression of 700 genes involved in the complement pathway and the innate immune response. Because maternal exposure to increased glucose appears to increase O-GlcNAc marks in the offspring, the children of diabetic mothers may be placed at increased risk for obesity and diabetes, which could result in a "vicious cycle" of increased risk for diabetes and metabolic syndrome perpetuated across generations.

NOVEMBER 14, 2008

SESSION 3: IMPRINTING, DEVELOPMENTAL PROGRAMMING, AND TRANSGENERATIONAL INHERITANCE OF EPIGENETIC MARKS

Epigenetics, Imprinting, and Disease Susceptibility

Randy Jirtle, Ph.D., Duke University Medical Center, Durham, NC

Children of famine survivors provide evidence for fetal origins of adult disease susceptibility. Children born after the Dutch famine of 1944-1945 had low birth weights and increased incidence of cardiovascular disease, obesity, diabetes, and schizophrenia as adults. During a severe famine in China, 50 percent of pregnancies did not result in live birth, and children born from the surviving pregnancies had a 2-fold increase in schizophrenia. These examples show that nutritional disruption early in development can lead to an increased susceptibility to adult diseases that can be transmitted to the next generation.

Epigenetics play a role in transmitting information and biological experiences across generations. Two classes of epigenetically labile genes exist: (1) imprinted genes, which are expressed from either the maternal or paternal allele in a regulated manner, and (2) metastable epialleles, which show highly variable expression because of stochastic allelic changes in the epigenome. Approximately 1 percent of the human genome consists of imprinted genes. Because of imprinting, maternal and paternal genomes are not functionally equivalent and both are needed for normal development. Creation of an embryo with two female pronuclei results in a small embryo with almost no placenta, while combining two male pronuclei results in a very large placenta, but no embryo.

Imprinting results in monoallelic, parent-of-origin dependent gene expression. In humans, only the paternal allele of *IGF2* is expressed, and only the maternal allele of H19 is expressed. The upstream region of *H19* is methylated in sperm, but not in egg. Methylation at the CTCF binding site in this region prevents binding of this transcription factor and activation of *H19* expression from the paternal allele. The lack of two functional alleles means that imprinted genes need only a single genetic or epigenetic event to dysregulate their function; imprinted genes thus are candidates for causative roles in human disease that have a parental inheritance bias and an environmental component in their etiology. Imprinting appears to be evolutionarily deleterious, but may be a remnant of the battle to balance the nutrient needs of the fetus and the mother. Imprinting in males tends to inactivate anti-growth genes, while imprinting in females tends to inactivate pro-growth genes; this difference may be related to the relative amounts of energy each sex expends on reproduction. The first evidence of imprinting dates back 150 million years and appears to have arisen in a common ancestor of monotremes and eutherians, although imprinting does not occur in monotremes. Imprinting at the *IGF2R* locus is observed in many animals, but not in humans or some other primates and appears to have been lost 75 million years ago. In 1995, loss of function of IGF2R was observed in a number of cancers, including liver and breast. Because of differences in imprinting at this locus in mice compared to humans, mice may not be the most appropriate model for studying human susceptibility to cancer.

The viable yellow agouti (A^{vy}) mouse provides an example of the impact of metastable epialleles on susceptibility of offspring to adult diseases. Approximately 60 percent of the offspring of A^{vy} mothers are yellow and obese if the mother was fed normal chow during gestation; if mothers are supplemented with folic acid or other methyl donors, 50 to 60 percent of the offspring are brown and of normal weight. The A^{vy} locus includes an intracisternal A particle (IAP) transposable element upstream of the agouti gene, resulting in an alternative cryptic start site for this gene. The A^{vy} coat color is dependent on methylation at the upstream promoter; complete methylation results in a brown coat, while lack of methylation results in a yellow coat. Variation in coat color and susceptibility to obesity is dependent upon the point of cell division at which the IAP region became methylated. The A^{vy} mouse can serve as an "*in vivo* biosensor" to assess the impact of the environment on the epigenome. For example, feeding pregnant A^{vy} mice bisphenol A resulted in hypomethylation and increased numbers of yellow offspring who were more likely to develop diabetes, obesity, and cancer. The lack of methylation occurs at this locus throughout the entire body and appears to impact the satiation center because these animals eat to excess. The effects of bisphenol A can be reversed by supplementations with methyl donors.

Future objectives in epigenetics include genome-wide prediction of imprinted genes. At present, a computational algorithm to identify imprinted genes has detected approximately 600 such genes in mice and 156 in humans, with an overlap of approximately 30 percent. The predicted imprinted genes function in organ development, including development of the nervous system, heart, kidney and lung. Approximately 50 percent of the predicted imprinted genes are involved in cancer and 3 genes have been mapped onto regions implicated in schizophrenia. A new genetic syndrome, Birk-Barel syndrome, has been defined based on identification of a mutation in a predicted imprinted gene.

Epigenetic Transgenerational Actions of Endocrine Disruptors on Reproduction and Disease: The Ghosts in your Genes

Michael K. Skinner, Ph.D., Washington State University, Pullman, WA

Transgenerational effects of environmental toxicants (e.g., endocrine disruptors) significantly amplify the impact and health hazards of these compounds. One of the most sensitive periods to endocrine disruptor exposure is during embryonic gonadal sex determination when the germ line is undergoing epigenetic programming and DNA re-methylation. The model endocrine disruptors tested were vinclozolin, which acts as an anti-androgenic compound, and methoxychlor, which has metabolites that are estrogenic. Previous studies have shown that these endocrine disruptors can effect embryonic testis development to subsequently cause an increase in spermatogenic cell apoptosis in the adult. Interestingly, this spermatogenic defect is transgenerational (F1, F2, F3, and F4 generations) and is hypothesized to be due to a permanent altered DNA methylation of the germ line. This appears to involve the induction of new imprinted-like DNA methylation sites that regulate transcription distally. The expression of more than 200 genes was found to be altered in the embryonic testis, and surprisingly this altered transcriptome was similar for all generations (F1-F3). In addition to detection of the male testis disorder, as the animals age transgenerational effects on other disease states were observed, including tumor development, prostate disease, kidney disease, and immune abnormalities. Recent observations suggest transgenerational effects on behaviors such as sexual selection and

anxiety. Therefore, the transgenerational epigenetic mechanism appears to involve the actions of an environmental compound at the time of sex determination to alter the epigenetic (i.e., DNA methylation) programming of the germ line that then alters the transcriptomes of developing organs to induce disease development transgenerationally. The suggestion that environmental factors can reprogram the germ line to induce epigenetic transgenerational disease is a new paradigm in disease etiology not previously considered.

Programming Central Circuits Controlling Feeding and Metabolism
Richard Simerly, Ph.D., University of Southern California, Los Angeles, CA

The arcuate nucleus of the hypothalamus (ARH) is a principal monitor of leptin signaling in the brain. Recently, we demonstrated that neural projections from the ARH to other parts of the hypothalamus develop during a postnatal period when leptin levels are elevated and that ARH projection pathways are severely disrupted in ob/ob mice. Leptin treatment in adulthood does not restore the normal pattern of projections, but the pathways are largely rescued by neonatal leptin treatments. Thus, the postnatal leptin surge appears to be a key developmental signal affecting the architecture of hypothalamic circuits mediating feeding during a discrete developmental critical period. Consistent with the concept that normal development of ARH projections is required for sensitivity to the weight reducing actions of leptin is the observation that rats susceptible to diet-induced obesity display central leptin resistance and have diminished ARH projections, compared to rats that are resistant to diet-induced obesity.

Leptin primarily signals through the long form of its receptor (LRb) and the trophic action of leptin on development of ARH projections appears to be mediated by this receptor. The LRb is expressed in the ARH during the developmental critical period, and leptin treatment activates a variety of cellular signal transduction pathways in neonatal mice, including phosphorylation of STAT3 and ERK1/2. In vivo axonal labeling of ARH projections in transgenic mice carrying LRb mutations that specifically impact these signaling pathways, together with pharmacological experiments with organotypic explant cultures, demonstrate the importance of JAK/STAT and MAPK signaling pathways for the trophic action of leptin on the development of ARH projections in the hypothalamus. Moreover, these signaling pathways appear to differentially affect NPY and POMC containing neurons, and neuropeptide expression in ARH projections displays considerable developmental plasticity.

Several studies in rats reported that alterations in litter size lead to marked changes in neonatal nutrition with concomitant changes in circulating levels of leptin (see Plagemann et al., 1999). We used a similar approach to effect postnatal alterations in nutrition and leptin secretion in neonatal mice. A marked increase in leptin levels was found in overfed compared to normal and underfed pups, whereas underfed animals exhibited a reduction in leptin levels compared to normal fed pups. Rapid catch-up weight gain was associated with postnatal undernutrition and these animals showed dramatic increases in both visceral and subcutaneous adiposity. Moreover, mice derived from small litters showed impaired glucose tolerance, and neonatally undernourished and overnourished mice both showed insulin resistance. Consistent with our findings in ob/ob mice, ARH projections in mice derived from large litters appear abnormal, suggesting that central defects in neural systems controlling behavior and autonomic regulation

may contribute to undesirable changes in energy balance. Collectively, these data show that early nutrition influences postnatal leptin secretion and suggest that it has long-term consequences on leptin sensitivity and brain development.

Prenatal Origins of Adult Disease

Peter W. Nathanielsz, M.D., Ph.D., D.Sc., University of Texas Health Science Center, San Antonio, TX

Developmental, or fetal, programming refers to the response to a specific challenge during a critical developmental time window that alters development qualitatively and/or quantitatively with resulting persistent effects on phenotype. The Barker Hypothesis proposes that events occurring during prenatal life have effects, particularly cardiovascular, on the adult. The fetal development phase is a particularly important time because humans pass more biological milestones before birth than any other time their lives, and how those milestones are passed can impact adult health. In humans, poor maternal nutrition or maternal stress have been observed to have effects on children that are evident in adulthood. To further explore the mechanisms by which developmental programming can impact the adult, animal models are needed.

Animal models for studies of developmental programming include rats, sheep, and primates. Studies in rats have shown that restricting protein by 50 percent (while maintaining calorie levels) during gestation results in obese offspring. Sheep also present a useful model for fetal development that allows chronic, longitudinal evaluation of the fetus; it also is possible to manipulate the fetus (i.e., place sensors) in the uterus and still have gestation reach full term. Global nutrient restriction (50% decrease in calories) during gestation results in heavier offspring with insulin resistance. By puberty (8 to 9 months of age) the pancreas of these sheep begins to fail and they develop diabetes. Glomerular number also is decreased in the calorie-restricted offspring and they develop hypertension by 8 months of age. Obese mothers and their fetuses also develop increased cortisol levels by mid-gestation. Fetal glucocorticoids directly affect normal development, particularly cardiovascular maturation, which is crucial for all developing organs. Experimentally decreasing cortisol levels in late fetal life ablates the normal increase in fetal blood pressure and decrease in heart rate that occurs during the last 25 days of gestation as part of the maturation process; replacing cortisol results only in a slight rise in heart rate.

Glucocorticoids alter the paracrine regulation of vascular tone in the fetus by altering the balance and integration of endothelin (ET-1) and NOS function. Responsiveness to ET-1 in the presence and absence of the NOS inhibitor L-NAME was examined in femoral resistance arteries from fetal sheep at 119 days of gestation following *in utero* exposure to maternally administered dexamethasone, given in a pattern resembling its use to prevent preterm labor. Each injection of dexamethasone resulted in an increase in fetal blood pressure, imposed a greater sensitivity to ET-1, and abolished nitric oxide (NO) release. This demonstrates that fetal exposure to inappropriate glucocorticoid levels increases ET-1 sensitivity in resistance arteries; ET-1 induced NO production was attenuated and direct ET-1 constrictor action was enhanced. By 18 months of age, there is no significant change in the exposed sheep compared to controls, but by 3 years of age, maximum tension and sensitivity to ET-1 are increased. Blood pressure also was higher, although not significantly, in adult sheep exposed to glucocorticoids as fetuses. The magnitude

of the increase was equivalent to an increase in blood pressure that increases risk of coronary artery disease by 20 percent in humans.

The effects of maternal nutrient restriction also have been studied in the baboon. A 30 percent nutrient restriction during gestation results in decreased tubular space in the fetal kidney, although no decrease in nephron number, implying shorter, less coiled tubules. The effects of moderate global maternal nutrient reduction on fetal baboon pancreatic development also were examined. By the end of gestation, the offspring from the calorie-reduced pregnancies showed a significant decrease in the number and size of the pancreatic islets. Staining for IGF-2 and insulin showed no difference halfway through gestation but a significant decrease in staining by 90 percent of gestation. Decreases in □1-adrenoreceptors (□1-AR) also were observed in the restricted offspring compared to controls. In contrast, nutrient restriction resulted in an increase in □2-AR levels halfway through gestation, but normal levels at term. These results imply that a relatively modest decrease (30%) in maternal nutrients increases the activity of the fetal gluconeogenic systems. Phosphoenolpyruvate carboxykinase (PEPCK) immunoreactivity was significantly increased halfway through gestation in the restricted animals and methylation of the *PEPCK* gene also was decreased in the kidneys of the offspring of restricted mothers halfway through gestation but reached normal levels by 90 percent of gestation. No changes were observed in liver and methylation levels were increased in brain only at late gestation. Both maternal and fetal cortisol levels also are increased in nutrient-restricted animals.

The marked differences in gestation among rodents, sheep, non-human primates, and humans argue for the use of non-human primate species to better understand developmental programming. The rodent generates a much larger biomass of products of conception relative to maternal rate than primates. A rodent weighing 250 grams and carrying sixteen pups plus placenta is the equivalent to a woman carrying a 30 kilogram baby. Marked differences in maturation occurring during fetal life also exist, as do significant differences in maternal behavior and neonatal endocrinology and behavior.

Epigenetic Processes in Developmental Origins of Health and Disease (DOHaD)
Mark Hanson, M.A., University of Southampton, Hampshire, United Kingdom

The risk for common diseases attributable to genetics is low, but there is a heritable component. The developing epidemic of diabetes and obesity appears to have both genetic and environmental causes, and influences early in life can shape disease risk later in life and also impact future generations. Mitigating the diabetes and obesity epidemic also is complicated by the fact that individuals living in the same environment may have different risks of developing these conditions. The same waist-to-hip ratio was associated with higher rates of diabetes in South Asians than in Europeans. Birthweight also appears to affect the risk of developing diabetes or metabolic syndrome; infants weighing 5.5 lbs at birth had a 30 percent chance of developing metabolic syndrome whereas infants weighing 7.5 lbs had an 18 percent chance. Aspects of body composition at birth appear to be associated with metabolic abnormalities late in life and certain environmental exposures can mimic this effect. The Pune Maternal Nutrition Study found that infants born with low birthweights did not have proportionally less fat, but instead had

less skeletal muscle and more abdominal fat than normal weight infants. These are characteristic risk factors for diabetes and metabolic syndrome.

Epigenetics underlie the effects of a poor developmental environment on adult health. Research has found that a low protein diet induces, while folic acid prevents altered epigenetic regulation of a number of genes relevant to metabolism. Methylation at the peroxisome proliferator-activated receptor-gamma (*PPAR* . and *AOX* promoters was decreased in the offspring of undernourished female rats, but expression was increased. This can produce permanent effects on the liver, even if birthweight is not affected. Supplementation with folic acid prevents changes in methylation and downstream effects. Protein restriction may lead to changes in promoter activity by decreasing binding of the maintenance methylase DNMT1 to promoters. No alteration in the binding behaviors of the *de novo* methylases DNMT3a or 3b was observed. Hepatic *PPAR* and *GR* promoter methylation was lower in the restricted group, and *PPAR* , *GR*, and *PEPCK* expression was higher in both the F1 and F2 males.

Feeding a high fat diet to the offspring of undernourished rats after weaning results in a dramatic increase in weight gain (attributable to increased food intake) compared to offspring of normally fed mothers on the same diet. Early postnatal leptin treatment can alleviate the obesogenic effects of this diet in the offspring of the undernourished mothers. Leptin treatment from postnatal day 3 to 13 resulted in slowing of neonatal weight gain and normalized caloric intake. Leptin treatment during early development could be modifying the developmental cues and re-setting the animals' appetites and metabolic regulatory systems to enable them to cope with the high nutrition environment. Maternal diet can alter the methylation pattern of specific CpGs in the *PPAR* promoter; this pattern persists into adulthood and predicts expression. *PPAR* expression and methylation patterns in liver are reset by leptin to resemble those of the offspring of rats fed a normal diet. Alterations in acetylation and methylation of histone proteins also were observed.

Nutritional alterations during pregnancy may have transgenerational effects. *GR* promoter methylation was lower, and expression higher, in rats born to mothers fed a protein-restricted diet during pregnancy. Analysis of the *GR* promoter in human umbilical cord blood found that methylation of this promoter was varied, but inversely related to *GR* expression. There was also a direct correlation between expression of *DNMT1* and promoter methylation. It may be possible to use these marks to draw inferences about intrauterine life, rather than using birth weight.

During the Dutch Hunger Winter of 1944 to 1945, women exposed to the famine in late pregnancy had smaller babies, while those exposed during early pregnancy did not, but their offspring developed features of metabolic syndrome as adults. Increased methylation at the IGFR locus was observed in the offspring exposed to famine during late pregnancy. The patterns set during intrauterine life have had transgenerational effects, with members of the F2 generation also having increased adiposity and poor health later in life. Based on work in the A^{vy} mouse model, it is possible that interventions early in life, for example supplementation with methyl donors such as folate, vitamin B, or choline, could reverse the effects of a nutrient-restricted intrauterine environment in humans. There also is early evidence that statins and vitamin D may have beneficial effects.

Epigenetics and Complex Disease: From Etiology to…
Arturas Petronis, M.D., Ph.D., University of Toronto, Toronto, ON

Identification of genetic and environmental causes of complex diseases, such as schizophrenia, obesity, diabetes, cancer, among numerous others, seems to be a much more complicated task when compared to cloning the genes in simple Mendelian conditions. The slow progress in research of complex diseases could be due to limitations of the basic strategy. I will discuss the results of two recent experimental projects performed in our laboratory. The first one is dedicated to twin studies and is related to the molecular basis of heritability. Traditionally, phenomenological twin studies provided the basis for genetic and epidemiological studies in human complex diseases. As epigenetic factors can contribute to phenotypic outcomes, we performed a DNA methylation analysis in white blood cells (WBC) and buccal epithelial cells of nearly 100 sets of monozygotic (MZ) and dizygotic (DZ) twins using the 12K CpG island microarrays. An intraclass correlation (ICC)-based comparison of matched MZ and DZ twins revealed significantly higher epigenetic difference in buccal cells of DZ co-twins. Although such higher epigenetic discordance in DZ twins can result from DNA sequence differences, our in silico SNP analyses and animal studies favour the hypothesis that this is due to epigenomic differences in the zygotes, suggesting that molecular mechanisms of heritability may not be limited to DNA sequence differences.

The second group of experiments has been dedicated to the elucidation of the molecular epigenetic basis of major psychosis, a prototypical human complex disease. Theoretically, we argue that in comparison to DNA sequence-based factors, epigenetic changes are more consistent with the non-Mendelian aspects of complex diseases. Experimentally, a CpG island microarray-based DNA methylation profiling revealed a number of epigenetic differences in the brains of individuals affected with major psychiatric disease vs. controls (N = 98) that were verified using the bisulfite sequencing. The same principles and strategies can be applied to various other complex non-Mendelian diseases, including diabetes, obesity, and metabolic syndrome.

X-Chromosome Inactivation: The Role of Noncoding RNAs in Directing Chromatin Change
Jeannie T. Lee, M.D., Ph.D., Harvard Medical School, Boston, MA

X-inactivation equalizes the X-chromosome dosages between the sexes. The X inactivation center (XIC), which serves as the master locus on the X-chromosome, covers approximately 100 kb in mice and contains only the noncoding RNA Xist and its anti-sense partner, Tsix. Xist is transcribed only from the inactive X and initiates the silencing process as it coats the X-chromosome; Tsix represses Xist. Both sense and anti-sense RNA is expressed from the X chromosome in undifferentiated embryonic stem cells. After the X to inactivate is chosen, expression from this chromosome is downregulated and Xist recruits silencing factors. Tsix expression persists on the active X chromosome until Xist expression is turned off. Xist is expressed at low levels from both X chromosomes before differentiation, and afterwards it is monoallelically upregulated.

Xist expression directs a cascade of chromatin changes in the X chromosome, including acetylation of the N-terminal lysine of histone H4 and hypermethylation of H3K27. Determining the mechanism by which Xist RNA initiates X chromosome silencing has been challenging because of difficulties with isolating Xist interacting factors. Previous research has shown that a transient heterochromatic state exists at the 5' end, leading to a wave of heterochromatin through the Xist locus on the inactivated X just before Xist is induced to spread across the chromosome. On the active X, the presence of euchromatin may repress Xist activation.

Polycomb repressor complex 2 (PRC2) and H3K27me3 can be observed at the 5' end of the Xist locus. The PRC2 complex contains a catalytic subunit, enhancer of zeste homolog 2 (EZH2) that methylates H3K27. PRC2 generally represses gene expression and has genome-wide regulatory effects during development that occur across species. Native state RNA immunoprecipitation using antibodies against PRC subunits followed by RT-PCR showed that Xist RNA associated with EZH2 and SUZ12, but only at its 5' end. Once cell differentiation begins and X inactivation commences, the rest of the Xist locus can be immunoprecipitated. In male cells, Xist binds EZH2 and SUZ12 before differentiation; after differentiation, the interaction decreases.

Xist directly recruits PRC2 to the X chromosome during X inactivation, and its recruitment correlates with H3K27 trimethylation across the entire X chromosome. PRC2 appears to preferentially bind the 5' end of Xist in a region called the Repeat A (RepA) motif. This motif has 6 to 7 tandem repeats of two stem-loop structures and is required for silencing, but not for RNA to coat the chromosome. Deletion of RepA prevents X inactivation, but Xist continues to coat the chromosome. Using RT-PCR, a 2- to 3-fold increase in RNA around RepA compared to the rest of the Xist locus was found. RepA produces a non-coding 1.6kb sense strand RNA in the same orientation as Xist. RepA is expressed from both X chromosomes before X inactivation; during X inactivation, one allele is lost, and the other becomes the inactive X.

To determine if PRC2 is recruited by RepA RNA, doxycyclin-inducible transgenic cells carrying Rep A on an autosome were created. Induction of the transgene with doxycyclin induces the RepA transgene 2- to 3-fold, which was associated with increased amounts of EZH2 and SUZ12, demonstrating that RepA is sufficient to recruit PRC2 to an ectopic locus and that recruitment depends on Rep A transcription. Further analyses found that both single-stranded and double-stranded RepA RNA associates with PRC2. Strand-specific RNA immunoprecipitation found equivalent amounts of sense and anti-sense RepA in the PRC2 complex.

Gel mobility shifts showed that RepA and Tsix RNA bind PRC2 directly. However, only EZH2 and not embryonic ectoderm development (EED) binds both RepA and Tsix, implying that EZH2 is the RNA binding unit; EZH2 has both methylation and RNA binding activity. The experiments also show that PRC2 can incorporate either sense or anti-sense RNA. Because the anti-sense Tsix transcript is a negative regulator of Xist, it could prevent X inactivation by competitive inhibitory interactions with Ezh2 or could prevent the preassembled complex from docking with the X chromosome. Xist recruits PRC2 to the inactivation center; this is followed by expression of Tsix, which prevents PRC2 from recognizing the X chromosome. Only upon

differentiation and loss of Tsix does spreading of Xist across the chromosome, and thus X inactivation, proceed.

To determine if RepA functions during X inactivation, silencer RNAs against RepA and another region of Xist were used to knock down expression. Only a few cells with knocked down RepA upregulated Xist RNA, as did cells in which other regions of Xist were knocked down. These results imply that loss of RepA impairs upregulation of full length Xist expression and X inactivation. Upregulation of Xist also was compromised in cells with knocked down EZH2 and EDd expression. Taken together, these results indicate that RepA and PRC2 are required for X inactivation in two ways. RepA and PRC2 induce a transient heterochromatic state in Xist that is required for Xist upregulation. After this, X inactivation is initiated by spreading of Xist across the X chromosomes and recruitment of PRC2 by RepA. Loss of the anti-sense transcript during X inactivation permits RepA and PRC2 to create the transient heterochromatic state that permits full expression of Xist.

SESSION 4: OF ENVIRONMENT, SEXES, AND FOOD

Growth Hormone Regulation of Sex-Dependent Liver Gene Expression

David J. Waxman, Ph.D., Boston University, Boston, MA

Sexually dimorphic patterns of gene expression in the liver may underlie the sexually dimorphic response to hepatic stress, leading to differences in conditions such as alcoholic fibrosis, hepatitis, and hepatocellular carcinoma in men and women. Genes expressed in a sexually dimorphic pattern in liver include the cytochrome P450 (CYP) genes, genes involved in steroid and drug metabolism, and phase II enzymes and transporters. These differences in gene expression are regulated by growth hormone (GH) secretory patterns; in males, GH is released in a pulsatile pattern while in females a steadier state of GH is maintained in plasma.

GH regulates sex-specific gene expression in liver primarily through STAT5b, a GH pulse-activated latent transcription factor, and HNF4□, a liver-enriched orphan nuclear receptor transcription factor and member of the steroid hormone receptor superfamily. GH stimulates STAT5b tyrosine phosphorylation, leading to dimerization and activation of transcription. Levels of phosphorylated STAT5b are relatively high in males, but deficient in females. Phosphorylation of STAT5b is driven by the GH pulses occurring in males; between pulses, STAT5b is unphosphorylated and retained in the cytoplasm. In *Stat5b* knockout mice, male-specific patterns of liver gene expression are not observed and the male animals do not undergo the typical pubertal growth spurt; female specific genes are up-regulated. Humans with mutations in *Stat5b* show a similar GH insensitivity. Knockout of *Hnf4* results in loss of the sexually dimorphic pattern of *CYP* gene expression. Expression of male-specific genes is suppressed in male knockout animals and some female specific genes are upregulated. Fewer differences are observed in female knockout animals. *Stat5b* or *Hnf4* knockout reduces or eliminates the sex-related differences in expression for approximately 90 percent of the more than 1000 sex-dependent genes expressed in liver. Male gene expression is lost, and between 42 and 61 percent of female-specific genes are de-repressed in the knockout males, demonstrating

that loss of function of these transcription factors has a significantly greater impact in male than female liver.

Male-specific transcriptional activators may be upregulated by the high levels of activated STAT5b and HNF4□ in males as primary targets and these then activate expression of secondary targets such as male-specific cytochrome P450s. The high levels of activated STAT5b in males also may activate transcription of male-specific female transcriptional repressors, which repress female-specific genes in male liver. Conversely, loss of STAT5b may lead to upregulation of female-specific transcriptional repressors, leading to loss of male-specific cytochrome P450 expression in STAT5b-deficient male livers.

High levels of STAT5b binding occur during GH pulses and low levels between pulses. Females have approximately 1 to 2 percent of peak male STAT5b binding activity. The lack of STAT5b binding between pulses suggests that STAT5b cycles on and off of chromatin. High levels of STAT5b binding is observed for some sites in both males and females, depending on the binding affinity of the STAT5b consensus site, which implies that sex-specific STAT5b binding sites will be of lower affinity and perhaps non-consensus.

GH pulsatility may regulate a small number of male-specific activators and repressors as evidenced by the slow response of certain cytochrome P450 genes to GH secretion. BCL6 is a male-specific transcriptional repressor and potential negative regulator of female-specific gene expression. *Bcl6* is suppressed transiently by the GH pulse and fully by the female-specific continuous pattern of GH secretion. Some, but not all, STAT5b binding sites also have BCL6 binding sites, which would enable BCL6 to selectively block STAT5b activation of these genes. Analysis of steady state levels of *Bcl6* mRNA in liver showed that the first several exons are expressed abundantly in female liver, but female transcription is selectively suppressed at intron 4, and is further suppressed at exon 5 (approximately 300-fold suppression). This region is only suppressed 6-fold in males, implying a female-specific transcriptional block in intron 4 and exon 5. In a model of STAT5b-BCL6 interaction to generate sex-specific gene expression, male-specific genes have low-affinity STAT5b sites and therefore bind STAT5b in male liver only; the site is not recognized by BCL6 and not subject to BCL6 repression in males. Female-specific genes have high-affinity STAT5b sites and thus bind STAT5b in both male and female liver; the site is recognized by BCL6 and subject to BLC6 repression in male, but not female liver.

CUX2/CUTL2 is a GH-regulated transcriptional repressor that is specifically expressed in females and can be induced in males given continuous GH. CUX2 binding sites are over-represented in the co-expressed set of STAT5b-dependent male genes compared to liver genes with sex-independent expression. Down-regulation of CUX2 at puberty precedes induction of male-specific CYP genes, including repressors of *Bcl6*; this leads to de-repression of female-specific genes, including activators of downstream genes. To explore the possible contribution of epigenetic factors to sex-specific gene silencing and the delayed response to GH of certain sex-specific CYPs, DNase I hypersensitivity experiments followed by tiling arrays were performed. Analysis of *Cyp7b1*, a male-specific CYP, found extensive hypersensitivity at this locus in males, but not females. Hypersensitivity was observed for female-specific genes,

implying that chromatin structure and potentially access to transcription factors also has a role in sex-specific gene expression.

Dietary Histone Deacetylase Inhibitors
Roderick H. Dashwood, Ph.D., Oregon State University, Corvallis, OR

The balance of histone acetylation and deacetylation activity is disturbed in cancer cells. Histone deacetylase (HDAC) inhibitors can induce growth arrest and differentiation in cancer cells and can halt tumor growth *in vivo*. Dietary HDAC inhibitors include butyrate, organosulfur compounds, and sulforaphane (SFN). Butyrate is produced during the metabolism of fiber and is the primary fuel of colonocytes. Allium compounds found in garlic have HDAC activity as do isothiocyanates and SFN compounds found in broccoli and broccoli sprouts. Many HDAC inhibitors have a conserved structure characterized by a cap region separated from the functional group by a spacer region of defined size. This structure permits them to fit into the HDAC pocket to inhibit activity.

The two major metabolites of SFN, SFN-cysteine and SFN-N-acetylcysteine (SFN-NAC) are effective HDAC inhibitors *in vitro*. Delivery of SFN-NAC by oral gavage to Apc^{min} mice, which spontaneously develop colon polyps, resulted in decreased HDAC activity in the colonic mucosa and increased acetylation of histone H3. Continuous delivery of SFN inhibits tumorigenesis and increases acetylation of histones H3 and H4. Crosslinking proteins to DNA followed by *in vivo* ChIP found increased acetylation of histones on the p21 promoter in the presence of SFN.

Exposing normal cells to HDAC inhibitors *in vitro* has no effect on growth, but prostate, colon, and breast cancer cells undergo apoptosis. Feeding SFN to mice retarded the growth of cancer xenografts; HDAC activity was inhibited and levels of acetylated histones were increased in the xenografts as well. Isolation of peripheral blood mononuclear cells (PBMCs) from animals treated with SFN found decreased HDAC activity in these cells. This is of interest because PBMCs are being used in clinical trials to monitor activity of the HDAC inhibitor SAHA. HDAC activity also was measured in blood of human volunteers fed broccoli sprouts, which contain 50 to 200 times more SFN than broccoli. A significant decrease in HDAC activity and induction of histone H3 and H4 acetylation was observed 3 to 6 hours after eating the broccoli sprouts; HDAC activity returns to normal within 24 hours. Thus, dietary HDAC inhibitors function in cells, animals, and humans.

The organosulfur compounds in garlic have been known to have anti-cancer activity. The garlic metabolite allyl mercaptan (AM) can fit into the HDAC pocket and inhibits HDAC activity *in vitro* in a dose-dependent manner. Dietary HDAC inhibitors are weaker ligands than clinical inhibitors; for example, 500mM AM is required to achieve a rapid, dose-dependent inhibition of HDAC, induction of histone acetylation, and growth suppression of human colon cancer cells, but produces a more sustained effect. AM arrests cells in G1 of the cell cycle and induces mRNA and protein of the cell cycle regulator p21 in a dose-dependent manner. ChIP studies of the p21 promoter found an increase in histone acetylation at this site. AM also increased Sp3, but not Sp1 binding, in the promoter. Four hours after AM administration, no changes was observed at a p53 binding site located in an upstream enhancer, but a significant increase in p53

binding was observed by 24 hours. Acetylation of the p53 locus also is of interest because acetylation status influences p53 gene expression.

Dietary HDAC inhibitors represent potential treatments for cancer, particularly given their relatively benign effects on normal cells. As weak ligands for HDAC, dietary HDAC inhibitors might subtly regulate genes (such P21 and BAX) that affect the response of normal cells to external stimuli such as oxidative stress or toxic insults.

Origins of Extreme Sexual Dimorphism in Genomic Imprinting
Déborah Bourc'his, Ph.D., Université Denis Didrot, Paris, France

The importance of biparental contribution to the genome is evidenced by the creation of embryonic lethal parthenogenotes and androgenotes in mice, each of which carry only maternal or paternal genomes, respectively. Maternal and paternal genomes are tagged by different epigenetic marks established during gametogeneisis. Establishment of specific maternal and paternal imprints is mediated by the *de novo* methyltransferases DNMT3a and 3L during gametogenesis. After fertilization, marks are protected and maintained by DNMT1, ZFP57, and Stella. Methylation in the male germline occurs before birth. In female germ cells, methylation commences only after the primordial germ cell reaches a size of 60 □M. Paternal marks occur pre-meiosis while maternal imprinting occurs after meiosis. Maternal post-meiotic germ cells are quadruploid, and have undergone meiotic recombination and changes in histone replacement and modification. Expression of *DNMT3L* is coordinated with imprint acquisition in both sexes. *DNMT3L* is expressed in fetal testes and seminiferous tubules and expression drops after birth. *DNMT3L* is expressed in oocytes after birth. Sex-specific recruitment of transcription factors to the *DNMT3L* promoter occurs to trigger expression in oocytes and spermatocytes.

In the mouse, 96 imprinted genes have been identified; 42 are maternally repressed and 54 are paternally repressed. These genes tend to be organized in clusters called imprint control regions (ICRs). More than 16 ICRs are controlled by maternal germline methylation and 3 are controlled by paternal germline methylation, suggesting underrepresentation of ICRs controlled by paternal methylation. In addition, many paternal ICRs have few CpG motifs and map to intergenic regions. Because it is established in stem cells before germ cell differentiation, the paternal germline must maintain its imprinting pattern for up to 3 years, versus approximately 20 days for maintenance of the maternal imprinting pattern in oocytes. Methylation sites also tend to be lost over time because of C to G transversions. However, the rate of CpG loss is not significantly increased at paternal ICRs, implying that differences in duration of paternal germline epigenetic marks are not responsible for under-representation of paternal ICRs.

To understand the impact of epigenetic marks in early development, maternal imprint-free embryos (with normal paternal imprints) and complete imprint-free parthenogenotes were created. Maternal imprint-free and complete imprint-fee embryos ceased to develop at e9.5, the point at which embryos become dependent on maternal resources. Microarray expression analysis of known imprinted genes showed that maternally expressed genes are dysregulated in the maternal imprint-free embryos and both maternal and paternal genes are dysregulated in the complete imprint-free embryos. Examination of the biological processes affected by maternal

imprinted genes, which include developmental information carried by the oocyte, found that processes for regulating implantation and development of the placenta were disproportionately affected. Abolition of the paternal germ line imprints did not rescue development in the imprint-free embryos, suggesting that paternal germ line imprints have a limited role in early development. Maternal germline imprints control genetic pathways needed for fetal-maternal interchange.

Sex- and Diet-Specific Epigenetic Marks
Claudine Junien, Ph.D., Dr. P.H., Hôpital Necker-Enfants Malades, Paris, France

Sex- and diet-specific epigenetic programming in the placenta is critical for resource allocation to the fetus and may also impact development of adult conditions, including obesity, metabolic syndrome, and diabetes. In humans, infants born at very low or very high birthweights (particularly to diabetic mothers) are at risk for developing diabetes. Approximately 50 percent of pregnant women in the United States are overweight or obese, and their offspring are at increased risk for diabetes even if born at a normal weight.

To examine the effects of diet on developmental programming, mice were fed a high fat diet from weaning to the age of 6 months. Approximately 20 percent of C57BL/6 mice will not become obese as a result of this diet because they will decrease their caloric intake; this is attributable to stochastic programming, because inbred mice are genetically identical. Only 10 percent of AJ mice show resistance to weight gain on the high fat diet, implying that a genetic component also has a role. Females who became obese on the high fat diet were crossed with normal males fed a control diet; the females were fed normal diets during gestation and lactation. At weaning, the offspring were placed on a high fat diet and 43 percent of the females were resistant to weight gain. When obese mothers were fed a control diet during gestation (although the mothers remained obese through gestation), more of the female offspring were resistant to weight gain. This suggests that adaptation of the diet during pregnancy may help slow transgenerational increases in obesity.

Among C57BL/6 and AJ mice fed a high fat diet, only C57BL/6 mice develop diabetes, and males develop the condition more frequently than do females, implying sex- and strain-specific differences in the development of metabolic syndrome and diabetes. The term placenta may carry markers indicative of nutrient conditions during pregnancy; the placenta could be considered both a nutrient sensing and nutrient supplying organ. Analysis of global genome methylation in the placentas of male and female offspring of mothers fed high fat or normal diets found hypomethylation in placentas of animals exposed to the high fat diet. Assessment of a cluster of imprinted genes involved in the maintenance of homeostasis in the amniotic fluid and the fetal circulation found differences in gene expression and methylation, but these differences were not solely attributed to methylation differences in promoter regions. Significant diet-related methylation differences were observed at specific CpG pairs in the differentially imprinted region (DMR), which overlaps the *Igf2r* locus on chromosome 17. The DMR contains consensus binding sequences for binding factors expressed in placenta. Comparison of gene expression patterns in males and females on high fat versus control diets identified 60 genes, including *Peg3*, *PPAR* , and *Cxc1*, that were differentially expressed, and more genes showed

altered expression in males fed high fat diets compared to females. These differentially expressed genes may function in pathways responsible for gender differences in fetal development and physiology and later in adulthood and could help identify molecular mechanisms that have both immediate and long-term effects on health.

Epigenetics, Brain Evolution, and Behavior: Co-adaptive Evolution of Imprinted Genes
James P. Curley, Ph.D., Columbia University, New York, NY

At least 80, and perhaps as many as 300, mammalian genes are expressed only from either the maternally or paternally inherited chromosome homologues. The majority of imprinted genes are found in specific regions of chromosomes in clusters that normally contain several protein coding genes and at least one non-coding RNA gene. Each cluster is generally under the control of a major *cis* acting element the ICR, which acquires differential methylation and other epigenetic chromatin modifications. The conflict hypothesis of imprinting evolution claims that paternally expressed genes would be growth promoting, thus favoring maternal investment in their own offspring at the expense of offspring of different fathers. Maternally expressed alleles would be growth suppressing, thus favoring maximization of the mother's reproductive potential over her entire lifespan. Some of the first imprinted genes discovered (i.e., *Igf2*), followed this pattern. Subsequently, imprinted genes have been demonstrated to regulate three groups of phenotypes: placental development, energy homeostasis and metabolism, and behavior.

A number of imprinted genes are involved in placental development. Knockout of paternally expressed genes results in a smaller than normal placenta, while knockout of maternally expressed genes results in a larger placenta. Imprinted genes also have roles in postnatal energy homeostasis. *Peg3* is a maternally imprinted allele that codes for a protein involved in regulation of p53-mediated apoptosis. *Peg3* knockout mice are smaller through their entire lifespan. At 1 month of age, these mice have less white adipose tissue (WAT) than control mice, but WAT levels normalize in later life despite the smaller size of the animals. The knockout mice also are hypophagic throughout life despite 10-fold higher plasma leptin level, indicating leptin resistance, but show no difference in glucose or insulin levels. The animals have lower energy expenditure, a lower core body temperature, and lower resting metabolic rate even accounting for lower body weight. *Peg3* knockout mice also are unable to increase their body temperature in response to the cold challenge, but can do so in response to a noradrenaline challenge, indicating that the brown fat is capable of adaptive thermogenesis in response to noradrenaline input, but the hypothalamus is apparently unable to trigger this. *Peg3* knockout mice have elevated levels of orexigenic peptide mRNA (*Npy*, *orexin*, and *MCH*) and reduced levels of anorexigenic *POMC* mRNA in the hypothalamus; this is a hyperphagic pattern, but the mice remain hypophagic throughout life.

Peg1/*Mest* is expressed only from the paternal allele and is widely expressed during embryogenesis in mesoderm-derived tissues and in the brain, particularly hypothalamus. Postnatal expression declines through lactation and is generally weaker in adult tissues. *Peg1*/*Mest* knockout mice are growth retarded and have a lower adult body weight and reduced levels of histologically normal WAT. *Peg1*/*Mest* expression is elevated in mice fed a high fat diet and expression was different in high versus low gainers. *Peg1*/*Mest* mRNA was

significantly increased in obese mice and in leptin knockout mice. The increased expression of *Peg1*/*Mest* in adipose tissue in obese mice was due to upregulation of the paternal allele, and not to loss of imprinting from the maternal allele. Higher levels of *Peg1*/*Mest* also were associated with enlarged adipocytes.

In humans, imprinted genes have been known to affect behavior, as in the case of Prader Willi and Angelman syndrome, both of which result from deletion of a segment of chromosome 15 but have very different phenotypes depending on whether the maternal (Angelman) or paternal (Prader Willi) allele is deleted. To study the effects of imprinting on brain development, chimeric mice were created. In parthenogenetic mice, cells with two copies of the maternal genome were preferentially distributed throughout the cortex and hippocampus. In androgenetic mice, cells with two copies of the paternal genome were preferentially distributed throughout the preoptic area, hypothalamus, olfactory mucosa, and vomeronasal organ. Knockout of *Magel2* (located on chromosome 15 in the Prader Willi cluster) results in mice with altered circadian rhythms and decreased levels of orexin A and B in the lateral hypothalamus, but elevated levels of their precursor. *Peg1*/*Mest* and *Peg3* knockout females have deficits in maternal care (i.e., slower or absent pup retrieval and nest building and reduced frequency of licking/grooming of pups) which are correlated with a reduced number of oxytocin-positive neurons and less oxytocin receptor binding. The wild type daughters of *Peg3* knockout females show behavioral changes such as inhibited exploration of novelty and their daughters also exhibited these differences. This transgenerational inheritance may be due to changes in the maternal behavior of the wild type daughters of the knockout females. *Peg3* knockout males exhibit deficient mating behaviors, including a lack of ability to discriminate between estrus and diestrus odors and show impairments in reproductive ability after 3 months of age.

Imprinted genes appear to function in control of embryonic resource acquisition, postnatal metabolism, and adult behavior. Imprinted genes are critical for normal development of metabolically relevant tissues and neuropeptide circuits in the hypothalamus and may be susceptible to environmentally induced changes in gene expression.