Linking Covalent Histone Modifications to Epigenetics: The Rigidity and Plasticity of the Marks

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In higher eukaryotes, genetic information, encoded by the DNA double helix, is organized into a complex chromatin structure with the assistance of histone and nonhistone proteins. As such, chromatin is the physiologically relevant form of eukaryotic genomes. While it is largely uncontested that different genetic compositions of individual species underlie their unique physiology and final morphology, chromatin provides an additional layer for dictating the translation of genetic information into meaningful biological readouts (for review, see Strahl and Allis 2000; Jenuwein and Allis 2001; Felsenfeld and Groudine 2003). Increasing evidence underscores an emerging theme in biology wherein “epigenetic” mechanisms, defined as mechanisms operating outside of changes in DNA sequence, govern gene function by creating potentially heritable alternative states of chromatin. DNA methylation and covalent modifications of histones, such as methylation, acetylation, and phosphorylation, are thought to contribute to these chromatin states by serving as a platform that integrates cell differentiation signals and environmental cues as well as regulating the timely release of the appropriate genetic information (Cheung et al. 2000; Schreiber and Bernstein 2002). Several of the above histone modifications are readily reversible through enzymatic means; others are potentially static (Bannister et al. 2002; see below). One central problem that remains to be resolved is how these mechanisms are regulated either during normal developmental conditions or under various pathological conditions.

EPIGENETIC MECHANISMS UNDERLYING DIFFERENTIATION DURING MAMMALIAN EMBRYONIC DEVELOPMENT

Gametogenesis gives rise to two different types of terminally differentiated cells, the sperm and the egg, which fuse during fertilization to initiate a new life cycle. During early developmental stages, the zygote and the blastomeres in two-, four-, and eight-cell embryos are totipotent, meaning that they possess an unrestricted developmental potential and are capable of differentiating into all cell types (Matzuk and Lamb 2002; Sassone-Corsi 2002). By the 16-cell stage, a gradual restriction in the developmental potential of cells takes place resulting in the production of two distinct lineages: (1) the trophoblast, which eventually develops into the extra-embryonic tissues; and (2) the inner cell mass, which develops into the embryo proper. The inner cell mass then gives rise to ectoderm, endoderm, and mesoderm. These different germ layers further develop and are organized to form various somatic tissues and organs (Fig. 1). This developmental process is associated with dramatic changes in gene expression profiles and is often referred to as cellular differentiation.

While mechanisms underlying the differentiation process remain unclear, several lines of evidence suggest the involvement of epigenetic regulation (as defined above; Kelly and Trasler 2004). For example, nuclear cloning experiments show that the developmental potential of a terminally differentiated somatic cell nucleus can be

Figure 1. Cell differentiation is based on epigenetic mechanisms. Differentiation, germ layer (ectoderm, endoderm, or mesoderm) formation, and organogenesis in a developing embryo are associated with heritable changes in gene expression profiles. However, the change of gene expression is caused not by the changes in the genetic information per se, but by epigenetic mechanisms. This notion is strongly supported by animal cloning experiments, which also suggest that eggs are rich with machineries capable of erasing the epigenetic information of a somatic nucleus.

fully restored to totipotency following transfer into an enucleated egg (Campbell et al. 1996; Wakayama et al. 1998). However, the low success rate of cloning (1–3% for adult somatic cell nuclei and 10–30% for embryonic nuclei) and the high rate of developmental defects associated with cloning strongly argue that epigenetic information carried by the transferred nucleus can be reprogrammed only on a limited basis (Hiendleder et al. 2004). On the other hand, these experiments suggest that poorly defined machinery stored in the egg is capable of reversing the biological clock back to its primary state. Identification of these suspected regulatory factors and the elucidation of their role during normal and abnormal developmental processes would offer important insights toward our understanding of epigenetic regulation of gene activity.

FUNDAMENTAL ROLES OF HISTONES IN ORCHESTRATING THE EPIGENETIC INFORMATION

The nucleosome is the basic structural unit of chromatin, consisting of about 146 bp of DNA wrapped around an octamer of core histones (including two each of histone H3, H4, H2B, and H2A) (van Holde 1989; Luger et al. 1997). Nucleosomal arrays are further stabilized by members of the linker histone H1/H5 family whose precise roles in chromatin function remains unclear (Wolffe 1998). While initially thought to be an inert structure involved in packaging DNA into the confines of the nucleus, significant progress has been made in documenting a more dynamic view of chromatin, particularly during transcriptional activation and repression (for review, see Felsenfeld and Groudine 2003; Khorasanizadeh 2004). This includes experiments with test “designer” genes where remarkable transitions between decondensed and condensed chromatin have been documented in living cells (Strukov et al. 2003; Janicki et al. 2004).

While there are many ways to introduce physiologically relevant variation into the chromatin polymer (Felsenfeld and Groudine 2003), numerous reports have documented that covalent histone modifications, working in a sequential and/or combinatorial manner, regulate DNA and chromatin templated cellular events, such as gene expression, DNA repair, and chromosome condensation (see Strahl and Allis 2000; Jenuwein and Allis 2001; Fischle et al. 2003). Well-documented histone modifications include acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation (for reviews, see Grunstein 1997; Cheung et al. 2000; Roth et al. 2001; Zhang and Reinberg 2001; Kouzarides 2002; Lachner et al. 2003). Other modifications, such as sumoylation (Shiio and Eisenman 2003), or the newly discovered citrullination (Hagiwara et al. 2002; this paper), are less well understood, but are likely to play key regulatory roles that will require further study. Although it remains unclear as to whether a true “histone code” exists, and, if so, the precise form and extent to which it is utilized (Agalioti et al. 2002; Schreiber and Bernstein 2002; Kurdiani et al. 2004; Schubeler et al. 2004), this

hypothesis offers a useful framework for the study of histone modifications, modifying enzymes, and effector proteins, as well as the role of these machineries and pathways in regulating important cellular functions.

HISTONE METHYLATION: STORIES OF Lys AND Arg

For the purpose of the discussion below, we highlighted several relevant modification sites in histone H3 and H4 in Figure 2A. Methylation of histone Lys residues, which can be mono-, di-, or trimethyl, is associated with actively expressed genes, as well as with silenced genes or large heterochromatin regions. For example, methylation at Lys4 and Lys79 of H3 is often found to be associated with actively expressed genes (Strahl et al. 1999; Santos-Rosa et al. 2003; Schubeler et al. 2004). On the other hand, methylation at Lys9 and Lys27 of H3 is closely correlated with the formation of repressive chromatin structure at both centromeric regions and silenced genes such as homoetic genes (for references, Schubeler et al. 2004; see below). Multiple histone H3 Lys9 methyltransferases have been reported, including G9a, Suv39h1, h2, and ESET (for review, see Lachner et al. 2003). Mice carrying mutations of these enzymes die at early embryonic stages (Peters et al. 2001; Dodge et al. 2004). EZH2, the well-studied methyltransferase of H3 Lys27, is also important for early mouse embryonic development (Erhardt et al. 2003). These results indicate that “setting” the epigenetic marks of histone Lys methylation ensures the normal embryonic development.

In addition to Lys methylation, protein arginine methyltransferases (PRMTs) modify multiple Arg residues in the amino-terminal tails of H3 and H4 (Fig. 2A). PRMT1 methylates histone H4 Arg3 residues, while histone H3 Arg17 is a preferred target site for CARM1 (Strahl et al. 2001; Wang et al. 2001; Bauer et al. 2002). Furthermore, methylation of Arg residues in H3 and H4 functions synergistically with histone Lys acetylation during activation of nuclear hormone regulated genes (Wang et al. 2001; Daujat et al. 2002). Recently, it was found that PRMT1, CARM1, and histone acetyltransferase p300 cooperate with p53 to activate gene expression (An et al. 2004). Taken together, these studies suggest that Arg methylation of histones is intimately involved in gene activation from chromatin templates.

CROSS-TALK OF DNA METHYLATION AND Lys METHYLATION IN HISTONES

For many years, DNA methylation, namely the 5-methylcytosine (5mC) modification at CpG islands of the genome, has been the main focus of the epigenetic gene regulation field (for review, see Jaenisch and Bird 2003; Feinberg and Tycko 2004). The findings that histone modifications can regulate DNA methylation patterns suggest that histone modifications, particularly Lys methylation, are important regulatory mechanisms of epigenetic phenomena, such as X-chromosome inactivation, imprinting, and cancer etiology. In *Neurospora crassa*, DIM5, a methyltransferase of histone H3 Lys9, mediates

Figure 2. Components of the epigenetic indexing system. (A) Covalent histone modifications, such as methylation of Lys and Arg residues, are marks associated with the genome to indicate gene expression potential of a chromatin region. These marks are “written” by enzymes that covalently modify histones. They exert their function by interacting with binding “effector” proteins, such as HP1, which “reads” H3 Lys9 methyl mark, and polycomb (Pc), which “reads” H3 Lys27 methyl marks. However, without clear binding effector proteins, methyl marks on some histone residues, including H3 Lys4, H4 Lys20, H3 Arg17, and H4 Arg3, remain as “orphan methyl marks.” (B) DNA methyl marks and histone methyl marks cross-talk with each other (see text for details). HMT, histone methyltransferase; DNMT, DNA methyltransferase.

DNA methylation (Tamaru et al. 2003). In *Arabidopsis thaliana*, KRYPTONITE, another histone H3 Lys9 methyltransferase, is also required for DNA methylation (Jackson et al. 2002). These studies suggest a regulatory mechanism whereby DNA methylation is targeted by histone methylation (Fig. 2B).

While the above-mentioned data supports the model that histone methylation guides DNA methylation, other reports suggest that DNA methylation may, in fact, regulate histone methylation as well. For example, DNA hypomethylation due to compromised activity of a maintenance DNA methyltransferase, MET1, causes defects in H3 Lys9 methylation in *Arabidopsis thaliana* (Soppe et al. 2002). Biochemical studies found that the methyl-DNA binding protein (MeCP2) interacts with H3 Lys9 methyltransferase (Fuks et al. 2003). These results suggest that there may be a continuous interplay between histone methylation and DNA methylation in certain biological systems. The possibility that DNA methylation may guide histone methylation is especially intriguing in the context of maintaining histone modification patterns following DNA replication.

BASIC CHARACTERISTICS OF EPIGENETIC MARKS: RIGIDITY AND PLASTICITY

Epigenetic gene expression patterns can be inherited by daughter cells following mitotic and sometimes even meiotic cell division (Cavalli and Paro 1999; Wolffe and Matzke 1999). Therefore, mechanisms must exist to es-

tablish and maintain these epigenetic marks in order to faithfully perpetuate gene expression patterns throughout cellular generations.

Establishing the Mark

What are the signals that establish an epigenetic state on a region of chromatin or on a particular gene? While some of the protein components involved in these pathways have been identified, it is less clear as to (1) how these marks are initially written onto the chromatin template and (2) how a multitude of epigenetic events are temporally coordinated and ordered. Moreover, the “trigger” of a given epigenetic event may vary from case to case.

In genomic imprinting, for example, the difference between two homologous alleles is often their developmental history (i.e., whether the allele is of maternal or paternal origin). During mouse embryonic development, the silencing of maternal or paternal X chromosome is random in the inner cell mass cells and their descendants. However, in the trophectoderm, the paternal X chromosome is selectively inactivated. Multiple epigenetic events, such as Xist RNA coating and changes in histone-modification patterns, are involved in these two X-inactivation processes (Okamoto et al. 2004).

On the other hand, in *Drosophila*, the formation of the dosage compensation complex is directly linked with the sex determination process. Translation of male specific lethal 2 (*msl2*) mRNA is repressed by the sex lethal

(SXL) protein in females (Kelley et al. 1997). Therefore, the MSL dosage compensation complex is formed to up-regulate the gene expression of the single male X chromosome by twofold. The mechanism of targeting the MSL complex remains poorly understood, but also involves production of noncoding RNAs and histone hyperacetylation (for review, see Park and Kuroda 2001).

In the case of establishing epigenetic marks on an individual gene, the transcriptional status of a given gene seems to determine the downstream epigenetic events. For example, in the process of homeotic gene silencing in *Drosophila*, the expression pattern is initially established by upstream transcription factors, which is then faithfully maintained in the subsequent development stages by the polycomb group of genes (for review, see Simon and Tamkun 2002). This finding underscores the interesting possibility that the absence of active gene transcription from a specific promoter may trigger downstream epigenetic events as witnessed by a recent study on $p16^{\text{INK4a}}$ (Bachman et al. 2003).

Maintaining the Mark

To impart their functions as epigenetic marks, histone modifications must be maintained during the cell cycle. The paradigm of how DNA methylation marks are propagated during DNA replication and cell division serves as a framework for a discussion on the maintenance of histone modifications, about which much less is known (Jaenisch and Bird 2003). Initial DNA methylation marks are generated by de novo methyltransferases, DNMT3a and DNMT3b. Since DNA methylation is often on the CpG islands of the DNA double helix, it can be inherited by semiconservative DNA replication. However, after DNA replication, the DNA methylation mark is absent from the newly synthesized daughter strands and therefore could be lost during subsequent rounds of DNA replication. To offset possible dilution effects, the maintenance methyltransferase, DNMT1, methylates the newly synthesized daughter strands of DNA according to the methylation patterns of the parental strands.

However, there are several significant differences in the way that histones are transmitted or inherited as compared to DNA methylation. First, although DNA is wrapped around histones to form the nucleosome, histones can be readily dissociated from DNA during the cell cycle. In extreme cases, such as mammalian spermatogenesis, the majority of histones are removed from the chromatin template and replaced by protamine (Sassone-Corsi 2002). Second, DNA methylation is retained on the parental strands after DNA replication, but histone modifications are distributed in a different manner: (1) In S phase, parental histones are distributed randomly onto the two newly duplicated DNA double helices (Wolffe and Matzke 1999 and references therein). Currently, it is not known whether the core histone octamer is incorporated as a whole entity or whether there is some dynamic change of the core histone components. Interestingly,

based on biochemical data in HeLa cells, a semiconservative nucleosome assembly model was recently proposed (Tagami et al. 2004). (2) Although the majority of histones are deposited into chromatin during S phase when DNA is replicated, interphase or transcription-associated histone replacement can also occur. For example, the histone H3 variant, H3.3, is actively incorporated into chromatin during interphase (Ahmad and Henikoff 2002).

In sum, mechanisms that govern the inheritance of histones and histone modifications are poorly understood. We suspect that incorporation of histone-modifying enzymes and binding effector proteins may serve to retain the pattern of chromatin marks in much the same way that spreading of heterochromatin, brought about by Lys methylation and HP1 binding, has been proposed to occur (Bannister et al. 2001). Determining how localized enzymatic activities recognize and propagate the patterns of covalent marks to nearby nucleosomes to compensate for the dilution effect of the cell cycle will be an important area of future investigation.

RESETTING EPIGENETIC MARKS IN GERM CELLS AND EARLY EMBRYOS

Dramatic resetting of the epigenetic marks occurs in germ line cells as well as in preimplantation embryos. DNA methylation marks are first erased and then regenerated during both male and female germ cell development (Hajkova et al. 2002; Lee et al. 2002). Shortly after fertilization, paternal DNA is actively demethylated by an unidentified DNA demethylase, while the maternal DNA methylation pattern is reset passively during subsequent cell divisions (Mayer et al. 2000; Li 2002). However, there are particular loci, especially the imprinted genes, that can escape active DNA demethylation.

Since most histones are replaced by protamine during spermatogenesis, it seems unlikely that the vast majority of "somatic" histone modifications can be transmitted to the next generation through sperm. The maternal genome in the egg, however, does appear to be decorated with a variety of histone modifications whose fate during early development is only beginning to be examined. To begin to test the hypothesis that the reprogramming of histone modifications may play a role in activation of the embryonic genome, we investigated global changes in levels of a series of histone tail modifications during oocyte maturation and preimplantation mouse development (Sarmiento et al. 2004). We found that certain "static" histone modifications, such as methylation of H3 Lys 9 and H3 Lys 4, appear to be relatively stable epigenetic marks during early development without exhibiting global change in our assays. However, other modifications, such as acetylation of H3 and H4 and methylation of H3 Arg 17 and H4 Arg 3, appear to be dynamically regulated. While the significance for the global resetting of histone Arg methylation marks remains unclear, we favor the view that this process may be involved in the dramatic changes in gene expression patterns observed during the egg-to-embryo transition.

From an epigenetic viewpoint, we favor the idea that the developmental life history of a cell is engraved on chromatin by posttranslational histone modifications and DNA methylation. If we expand upon and broaden this concept, then in fact all of the structural proteins and enzymes responsible for the generation and maintenance of these epigenetic modifications can also be considered as a part of the “epigenetic information.” For example, a certain structure formed on a chromatin region, such as condensed structures on pericentric heterochromatin or silenced homeotic genes, is inherited through cell cycles or memorized by the next generation. Thus, we would like to introduce the concept of “chromatin state,” in which DNA and histone modifications interact with their binding or “effector” proteins and affect the local chromatin conformation in order to accommodate different chromatin/DNA templated events, such as transcription (Fig. 4).

Differentiation would be a process of continually refining the chromatin state leading to different sets of gene expression. We can consider the differentiation signals or cues as input, which then leads to the fine adjustment of the chromatin state in a process of “editing” and “writing.” The adjustment of the chromatin state can bring about changes from state A to state B, leading to cell differentiation during embryonic development. Multiple machineries, such as the transcriptional complexes, would respond to this change of chromatin state, read the epigenetic information on chromatin, and translate it into meaningful biological readouts. Although differentiation has been considered to be a unidirectional commitment, recent data demonstrated that the forced expression of

C/EBP α and C/EBP β (transcription factors involved in hematopoietic cell differentiation) can reprogram B cells into macrophages, suggesting that the manipulation of upstream master control genes can dramatically change the chromatin state of one cell type into another, thus altering the differentiation pathway (Xie et al. 2004).

In the context of the cloning experiments, the chromatin state in a given cell type, such as a neuron, might be reprogrammed to the primary chromatin state (i.e., the zygote) from which all of the other chromatin states arise (Eggan et al. 2004). The mammalian egg’s ability to reprogram terminally differentiated nuclei suggests that eggs have all the machineries required to erase the historic “records” on histones and DNA. New proteomic techniques, combined with the completed human and mouse genome sequences, will likely allow us to identify many of the factors involved in these early steps of development. Ultimately, biochemical and genetic approaches should help us obtain an idea of how these protein components work together to orchestrate this dramatic reprogramming event.

HISTONE METHYLTRANSFERASES AND EPIGENETIC TUMORIGENESIS

The imbalance of stem cell proliferation and differentiation can lead to cancer. For example, in patients with acute promyelocytic leukemia (APL), promyelocytes keep dividing and fail to differentiate into granulocytes. The ability of all-trans retinoic acid (ATRA) to induce the differentiation of APL cells has offered a successful chemotherapeutic agent to treat APL patients for more than a decade (Fenaux et al. 1992). Therefore, changes of gene expression profiles and epigenetic histone modifications in response to ATRA switch a “cancer” chromatin state to another chromatin state of a short-lived granulocyte. In the following discussion, we will focus on the epigenetic aspect of tumorigenesis.

Enzymes that modify different Lys sites in H3 have numerous links with human cancers. For example, human MLL1 (also termed ALL1, HRX, or HTRX) has been shown to methylate H3 Lys4 (Milne et al. 2002; Nakamura et al. 2002). The translocations and fusions of MLL1 with dozens of other genes result in acute leukemia. It is intriguing to speculate that MLL normally functions as a tumor “epigenetic” suppressor (see Fig. 5). Chromosome translocations between the *ml* locus and many other loci result in the loss of the SET domain of MLL. Therefore, the set of genes epigenetically regulated by MLL through marking/indexing with H3 Lys4 methylation is perturbed, which might lead to carcinogenesis. The lacking of the H3 Lys9 methyltransferases, Suv39h1 and -2, in mice leads to telomere abnormalities, chromosome instability, and increased rate of B cell lymphomas (Peters et al. 2001). Although the effect on chromosome instability and telomere length should be well considered, we speculate an epigenetic gene-regulation mechanism similar to that of MLL underlying the high incidence of B cell lymphoma. Interestingly, RIZ1, a Rb interacting H3 Lys9 methyltransferase, has been well characterized as a tumor suppressor

Figure 4. The concept of a chromatin state. The epigenetic information and the binding effector protein of epigenetic marks form an index system of gene expression potentials and regional chromatin conformation that determines a chromatin state. In response to extracellular cues (input), new epigenetic information is “written,” and chromatin state “A” can be modified to become chromatin state “B.” The change of chromatin state is translated (read) to cellular functions, which underlie(s) various processes such as cell differentiation, gene activity, and etiology of some diseases. Change(s) of chromatin state in the opposite direction (reprogramming) can happen during cloning or dedifferentiation.

Figure 5. Histone Lys methyltransferases and epigenetic cause of tumorigenesis. (A) In this model, MLL (targeting Lys4), RIZ1, and Su(var)3-9 (targeting Lys9) are considered as tumor “epigenetic” suppressors. Members of the E(z) pathway (EZH2, Bmi1) are considered as “epigenetic” oncogenes. (B) MLL marks the expression potential of genes (A, B, C) by methylation of H3 Lys4 in normal cells. Loss of the normal function of MLL by chromosome translocations causes the repression of these genes thereby inducing cancers. On the other hand, RIZ1 and Su(var)3-9 index the silencing of their target genes (D, E, F); the loss of the repressive marks (H3 Lys9 methylation) leads to overexpression of these genes, thus leading to carcinogenesis. In contrast, the E(z) pathway maintains the cell proliferation potential by silencing a set of genes (X, Y, Z). The ultra-active E(z) function can cause cancers either by super-silencing of its target genes (X, Y, Z) or by silencing a new set of genes (M, N), which is normally expressed to prevent cancer development.

(Kim et al. 2003). The low expression and point mutations of RIZ1 are correlated with various human cancers, including breast cancer, liver cancer, colon cancer, neuroblastoma, melanoma, lung cancer, and osteosarcoma, suggesting that RIZ1 may suppress tumorigenesis via an epigenetic mechanism to ensure gene expression profiles of normal cells (Steele-Perkins et al. 2001).

On the other hand, human H3 Lys27 methyltransferase EZH2 was often overexpressed in metastatic prostate cancer and Hodgkin and non-Hodgkin B cell lymphoma (van Kemenade et al. 2001; Varambally et al. 2002). Similarly, Bmi-1, the human homolog of *Drosophila* Pc, which was shown to regulate the proliferative capacity of normal and leukemic stem cells, is upregulated in cancer cells (Lessard and Sauvageau 2003; Molofsky et al. 2003). Therefore, the EZH2 complex may be overexpressed in cancer cells to hyperrepress cell cycle inhibitor genes, thereby promoting tumorigenesis in a manner like an “epigenetic” oncogene.

In summary, proteins controlling epigenetic gene expression are important regulators of normal cell division, and differentiation, as well as tumorigenesis. We and others favor the idea that histone- and chromatin-modifying enzymes represent excellent targets for exploring pharmaceutical compounds to counteract cancers and other epigenetic disorders (Huang 2002).

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