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Histone Lysine Demethylases and Their Impact on Epigenetics

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Methylation marks on the lysine residues of histone proteins are thought to contribute to epigenetic phenomena in part because of their apparent irreversibility. Will this view change with the recent discovery of histone lysine demethylases that reversibly remove methyl marks?

Chromatin establishes, maintains, and propagates different patterns of gene expression by storing and organizing genetic information. Histone lysine methylation has been regarded as a stable chromatin modification that together with DNA methylation defines epigenetic programs. Epigenetic phenomena are responsible for the non-Mendelian inheritance of phenotypic alterations. The recent discovery of histone lysine demethylases that reversibly remove methyl marks appears to challenge the epigenetic potential of histone lysine methylation. However, we argue that the reversibility of histone lysine methyl marks does not jeopardize their epigenetic status. We also suggest that not all histone lysine methylation residues are equally reversible and argue that two such residues—present exclusively in multicellular organisms—play important roles in establishing cellular identity.

The Complexity of the Histone Methylation Machinery

"Lower" eukaryotes (unicellular organisms) usually have a relatively short life cycle, a simple genome organization and a profile of gene expression that must adapt rapidly to environmental cues. An extraordinary step during evolution was the development of multicellular organisms with their increased genome size and complexity. This complexity required the transmission of "active" and "repressed" genetic information to the next generation. Multicellularity also necessitated that different cells of an organism became specialized for certain tasks. Therefore, a system had to evolve that would be capable of organizing cellular differentiation (and establishing cellular identity) through early development and that maintained a cell's given identity in adulthood. This system is found in the complicated machinery that regulates the chromatin of higher eukaryotes, in which the majority of chromatin is condensed (heterochromatin).

The number of methyltransferases that add methyl groups to lysine residues increases dramatically from lower to higher eukaryotes. Reflecting the difference in global chromatin organization, the number of methylation sites on the lysines (K) of histones (H) also increases from yeast to human. Methylation marks that are linked to open chromatin and transcriptional activation (H3K4, H3K36, and H3K79) are present in all eukaryotes. In contrast, enzymes targeting methylation sites characteristic of condensed chromatin and transcriptional repression (H3K9, H3K27, and H4K20) are not present in the budding yeast, *Saccharomyces cerevisiae*. Moreover, histone lysine residues can be mono-, di-, or trimethylated, and a picture has begun to emerge in which different degrees of methylation on one particular site could be linked to different functional outcomes. Generally, in lower eukaryotes all three degrees of methylation on a particular histone methylation site are regulated by the same enzyme, whereas in higher eukaryotes histone lysine methyltransferases have been identified that control only one degree of methylation. This adds an additional layer of complexity to the chromatin regulatory functions of histone lysine methyltransferases in higher eukaryotes.

Histone lysine methylation seems to be an anomaly among histone modifications. Contrary to other modifications, the global turnover of lysine methylation is low, suggesting that the modification is stable. Until recently, there was little doubt that stably methylated histone lysine residues contributed to the establishment and propagation of different patterns of gene expression in the same genome. Thus, methylated histone lysine residues have been considered "epigenetic marks" (Jenuwein and Allis, 2001).

Histone Lysine Methylation Is Reversible

Although researchers sought evidence for enzymatic demethylation of histone lysine residues, the technical difficulties in setting up an in vitro assay for this type of demethylase activity hindered progress. In the absence of evidence for histone lysine demethylases, the apparent loss of histone methyl marks

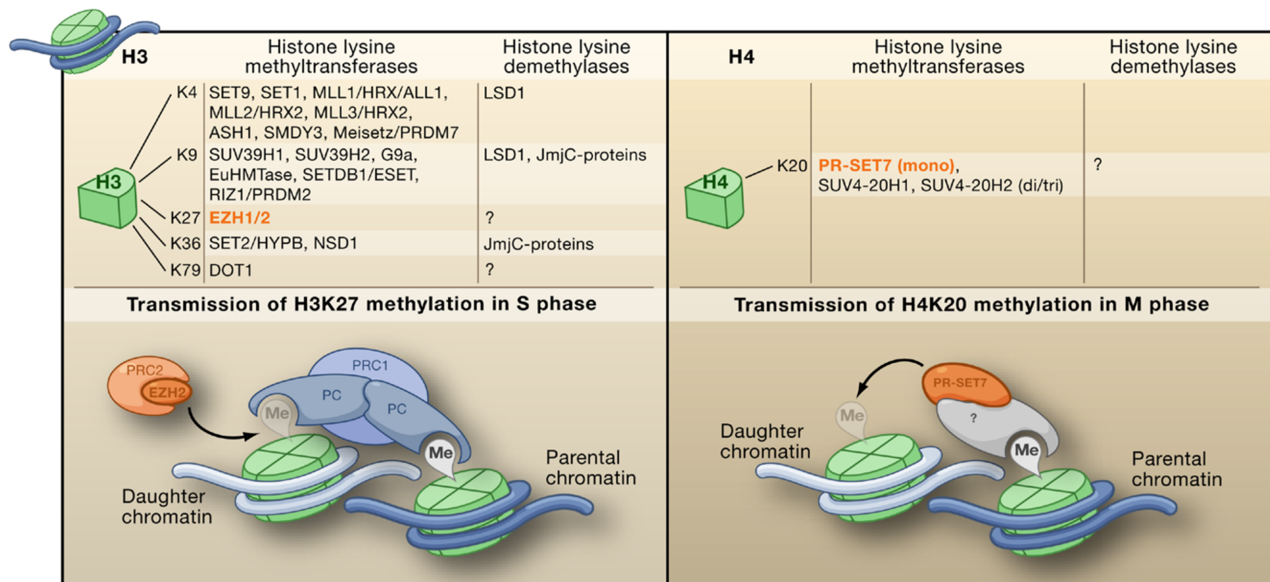

Figure 1. Methylation of Histone Lysines

(Top) The enzymes that methylate and demethylate lysine (K) residues of histones H3 and H4 are shown. In higher eukaryotes, methylation of H4K20 (H4K20me1) is catalyzed exclusively by the methyltransferase PR-SET7 (the SUV4-20H1/2 enzymes are responsible for H4K20me2/3). H3K27 methylation (H3K27me, mediated by EZH1/2) and H4K20me1 (an autonomous mark independent from H4K20me2/3) are marks not found in unicellular organisms but which rather appeared with the emergence of multicellularity. Histone demethylases of the LSD1/BHC110 family are absent in the budding yeast *Saccharomyces cerevisiae* but are present in the fission yeast *Schizosaccharomyces pombe*. Certain proteins containing Jumonji (Jmj) domains, which are conserved from yeast to human, have histone demethylase activity. DOT1 is the only enzyme responsible for methylating H3K79, a methyl mark that is associated with maintaining open chromatin. H3K79me is also present in *S. cerevisiae*, and so we speculate that the epigenetic potential of H3K79me is different from that of H4K20me1 and H3K27me. (Bottom) The transmission of the epigenetic histone methyl marks H4K20me1 and H3K27me from parental to daughter chromosomes. (Bottom, left) The model proposes that H3K27me marks are transmitted during DNA replication. A Polycomb dimer binds to H3K27me on the parental chromatin and an unmethylated H3 tail from the newly synthesized chromatin. EZH2, the H3K27-specific histone lysine methyltransferase, is recruited and methylates H3K27 on the daughter strand. (Bottom, right) DNA is replicated in S phase, but H4K20me1 is not transmitted to newly synthesized chromosomes before mitosis. PR-SET7, the H4K20me1-specific histone lysine methyltransferase, is only expressed during mitosis. The enzyme directly (or indirectly through interaction with an unknown H4K20me1 binding protein) recognizes H4K20me1 on the parental chromosome and methylates the appropriate position on the daughter chromosome.

was explained either by “passive” demethylation (that is, every round of replication “dilutes” the total number of modified histones) or by histone exchange. Identification of the first enzyme responsible for histone lysine demethylation changed this view.

A groundbreaking study reported the discovery of H3K4 demethylation by the amine oxidase family member LSD1 (Shi et al., 2004). This enzyme removes with remarkable specificity one or two methyl groups from H3K4 (H3K4me1/2) but cannot attack trimethylated H3K4 (H3K4me3). Surprisingly, LSD1 was also reported to demethylate H3K9 (H3K9me1/2) upon interaction with the androgen receptor (Metzger et al., 2005). (Several excellent review articles provide a detailed discussion of the chemistry and substrate specificity of LSD1 [Bannister and Kouzarides, 2005; Kubicek and Jenuwein, 2004]). Recently, it was proposed that a family of proteins containing Jumonji C (JmjC) domains demethylates histone lysine residues (Trewick et al., 2005), and an activity called JHDM1A that specifically catalyzes demethylation of H3K36 (H3K36me1/2) has been purified (Tsukada et al., 2005). The same group has purified a homologous JmjC protein that demethylates H3K9me2 resulting in unmodified H3K9 (Yamane et al., 2006). Meanwhile, others have discovered demethylation of H3K9me3: ectopic expression of the Jmjd2b protein resulted in a global reduction of H3K9me3 in vivo (T. Jenuwein, personal communication). Moreover, various JMJD2 proteins were found to antagonize H3K9me3 and H3K36me3 both biochemically and in vivo, resulting in H3K9me1/2 and H3K36me1/2, respectively (Whetstine et al., 2006). The mammalian Jmj protein family is large, and it is likely that there are other histone lysine demethylases that await discovery. The identification of histone lysine demethylases raises the possibility that all three degrees of methylation at these sites could be reversible.

Although the discovery of histone lysine demethylases would be predicted to impact the epigenetic potential of histone lysine methylation, we believe it does not. These are the reasons why: (1) demethylation of histone lysine residues does not change the fact that some methylation marks seem to be very stable and exhibit low turnover rates (at least in restricted regions of chromatin or in certain cellular states). Therefore, expression and/or activity of histone lysine demethylases must be tightly regulated in differentiated cells. (2) There is accumulating evidence that

DNA methylation is reversible yet its epigenetic potential is not in dispute. DNA methylation is dependent on histone lysine methylation in various cases (Tamaru and Selker, 2001). Therefore, histone methylation marks may influence epigenetic memory formation indirectly by regulating DNA methylation. (3) We still know very little about the biology of histone lysine demethylases. Many chromatin regulatory proteins are dynamic and are constantly recruited, bound, and ejected from chromatin. Are histone lysine demethylases also highly dynamic or are they stably bound to chromatin to preserve a demethylated state? (4) We need to assess the plasticity of histone methyl marks during development (for example, by genome-wide ChIP-on-chip analysis for each lysine methylation site and for all degrees of methylation). Moreover, analyses must be performed at different developmental stages (e.g., zygote, blastula, gastrula) necessitating technical advances to deal with such limited material. However, these techniques are not expected to detect epigenetic information that is restricted to limited but developmentally important regions of chromatin. (5) There is accumulating evidence that one methyl mark by itself might have only a limited biological message. For instance, H3K9me3, originally considered a hallmark of constitutive heterochromatin, was recently reported to be present at actively transcribed genes. Therefore, although H3K9me3 by itself might not correlate directly with transcriptional state, in combination with other histone marks, it might still contribute to the final outcome. In general, the spatial and temporal context of histone lysine marks seem to be important. (6) Thus far, only a small number of residues (H3K4me1/2, H3K36me1/2, and H3K9me1/2/3) are antagonized by a restricted number of demethylases (see Figure 1). It is likely that demethylases targeting other histone lysine residues will be discovered in the near future, but it is also possible that some histone methyl marks are not targeted by demethylases at all.

Transmission of Epigenetic Histone Lysine Marks

The crux of an epigenetic mechanism involves the transmission of information via the germline to the next generation of a multicellular organism. We still do not fully comprehend how histone methylation marks are maintained throughout the cell cycle, or how epigenetic marks are transmitted through the germline to the next generation. Although potentially every histone lysine methyl mark provides epigenetic information, here we provide a model of how two specific histone lysine methyl marks could be transferred to daughter cells.

The two methyl marks, H3K27me and H4K20me1, are each established by one enzyme, EZH2 and PR-SET7, respectively (other methyl marks may be established by several enzymes) (see Figure 1). EZH2 and PR-SET7 emerged at the same time as multicellularity. Moreover, H3K27me and H4K20me1 are not known targets of demethylases, and both marks are present on the inactive X chromosome. Transcriptional inactivation of the X chromosome is an excellent system with which to study epigenetic mechanisms, because one X chromosome must remain silent in all cell types and throughout all cell divisions of female mammals. We propose that H3K27me and H4K20me1 may have limited reversibility and could be used to transmit epigenetic information.

An important property for an epigenetic histone lysine methyl mark is that it has to be established and maintained throughout the cell cycle. Monomethylation of H4K20 fulfills several criteria for an epigenetic mark. The expression of PR-SET7, which monomethylates H4K20, is strictly regulated during the cell cycle, being detectable only during late G2 and early M phase. The H4K20me1 mark is present throughout the cell cycle, suggesting that it is not removed in interphase cells that do not have PR-SET7 to replace it. This also suggests that H4K20me1 is not antagonized by histone demethylation. Persistence of H4K20me1 was also observed in *Drosophila* embryos lacking *PR-Set7*. In these embryos, the maternally deposited modification is present until late larval stages, at which point defects in proper cell division appear resulting in lethality (Karachentsev et al., 2005). These data suggest that H4K20me1 is not erased by histone lysine demethylases during early embryonic development. Mitosis is the final stage for propagating epigenetic information from parental chromatin to newly generated chromatin prior to chromosome segregation and cell division. It has been proposed that the physical association of PR-SET7 with mitotic chromosomes places it at the appropriate position for transmitting H4K20me1 marks from mother to daughter cells (Reinberg et al., 2004). This epigenetic mechanism would necessitate that PR-SET7 somehow recognizes H4K20me1 on the mother chromosomes and then "writes" the same mark on corresponding positions on the daughter chromosomes (see Figure 1; D.R. and P.T., unpublished data).

Methylation of H3K27 is performed solely by EZH2, the mammalian homolog of the *Drosophila* protein Ez (enhancer of zeste). Ez is a member of the Polycomb (PcG) protein family and is crucial for the maintenance of transcriptional repression of the developmentally important homeotic (Hox) genes. Polycomb- and Trithorax-group proteins are known to be key regulators of proper embryonic development and are important players in maintaining cellular identity established early during development in multicellular organisms. The finding that PcG proteins stabilize long-term transcriptional silencing of homeotic genes provided the first evidence for a molecular mechanism of "cellular" or "epigenetic" memory (Rastelli et al., 1993). EZH2 executes its histone lysine methyltransferase activity only as a component of various multiprotein complexes ((Reinberg et al., 2004). Collectively, there is compelling evidence that EZH2 plays an important epigenetic role in establishing cellular identity and that this function is ultimately linked to its H3K27 histone lysine methyltransferase activity. Moreover, EZH2 directly interacts with DNA methyltransferases in vitro.

RNAi against EZH2 resulted in a loss of DNA methyltransferases and CpG methylation of target genes in vivo (Vire et al., 2005). Therefore, EZH2 also might have an indirect impact on epigenetic phenomena by regulating DNA methylation.

H3K27me3 is important in imprinted and random X chromosome inactivation (Heard, 2005). Imprinted paternal X chromosomes are loaded with H3K27me2/3 during preimplantation stages and are maintained in extraembryonic cells, but the methyl mark is then lost in the inner cell mass of the embryo at the blastocyst stage. These changes in the status of H3K27 methylation reflect a general plasticity of this modification and indicate that an H3K27-specific histone lysine demethylase might be expressed in embryonic cells. H3K27me3 is also observed during random X chromosome inactivation in embryonic stem cells upon their differentiation. Although the mark is dependent on the expression of Xist, a noncoding RNA that coats the inactivated X chromosome during the early stages of differentiation, at late stages the K27 methyl mark persists even when Xist expression is blocked (Kohlmaier et al., 2004). This corroborates the role of H3K27me3 as an epigenetic mark and suggests that either a putative H3K27-specific histone lysine demethylase is not recruited to the inactive X chromosome or that it simply does not exist in differentiating embryonic stem cells.

The mechanism by which the H3K27 methyl mark is transferred from mother to daughter chromosomes is still unclear. However, in contrast to the case of PR-SET7 where the factor recognizing and binding to H4K20me1 remains to be discovered, Polycomb has been identified as the binding protein for the H3K27me2/3 mark. Polycomb, a component of the PRC1 complex, binds as a dimer to two histone H3-tails simultaneously; structural studies demonstrate that the bound tails are in close proximity (Min et al., 2003). For steric reasons, it is unlikely that the two H3 tails come from the same nucleosome, and this may provide a mechanism whereby a Polycomb dimer (or PRC1) could compact chromatin by binding simultaneously to two nucleosomes. We speculate that this mechanism may transmit the epigenetic H3K27 methyl mark from parental chromatin to newly synthesized chromatin (see Figure 1). The PRC1 complex (and Polycomb) binds to unmodified (or tail-less) nucleosomes (Francis et al., 2004). In vitro binding studies of Polycomb and histone H3 tails suggest that H3K27 methylation only facilitates binding. Therefore, it is possible that the Polycomb protein can bind to unmethylated histone H3 tails. Given that transient interactions between the EZH2 complex and Polycomb have been reported (Poux et al., 2001), EZH2 may be recruited as the "epigenetic indexer" of Polycomb bound histone H3 tails of daughter chromosomes. Alternatively, DNA-specific binding factors like zeste or GAGA (components of PRC1) could mediate transient interactions between PRC1 and EZH2 complexes (Mulholland et al., 2003).

Which stage of the cell cycle then is selected to transmit the H3K27 methyl mark? EZH2 and its associated polypeptides are target genes of the E2F transcription factor, and many targets of this factor are expressed at the transition from G1 to S phase. Moreover, *ezh2* knockdown by RNAi causes severe defects in cell proliferation, suggesting a role in cell cycle progression. Recently, binding of the EZH2 complex and PRC1 to the inactivated X chromosome was shown to be highly dynamic and cell cycle stage dependent, occurring primarily in early- to mid-S phase (Hernandez-Munoz et al., 2005). Collectively, these studies suggest that, within the context of our proposed epigenetic mechanism for the inheritance of the K27 methyl mark, this mark would likely be placed during S phase immediately after DNA replication (see Figure 1). The major issue is our limited knowledge regarding how Polycomb-group proteins become recruited in the first place. In *Drosophila*, Polycomb-responsive elements (PRE) constitute specific DNA sequences located several kilobases upstream of the transcriptional start sites of the *Hox* genes. These PREs are targeted by Polycomb-group proteins that bind to DNA and then recruit the EZH2 complexes and PRC1. Surprisingly, repression by PREs can affect genes over wide distances in the genome, and these elements somehow seem to interact with each other. These remarkable findings have led to a model during replication in which the PRE bound Polycomb-group complexes loop toward and bind to the PREs on newly synthesized daughter chromosomes, thus propagating the epigenetic marks (Pirrotta, 1998). However, PRE sequences have not been found in mammals as yet, and so this model cannot explain the recruitment of EZH2 complexes to chromatin in mammalian cells.

Conclusion

We suggest that two histone lysine methyltransferases, EZH2 and PR-SET7, are important epigenetic regulators given that their specific substrate residues, H3K27me and H4K20me1, remain stably methylated over several cell generations, at least in particular chromatin regions. EZH2 and PR-SET7 emerged with the appearance of multicellularity and a complex system to regulate cellular identity. We propose that H4K20me1 and H3K27me are not erased by histone lysine demethylases, at least not at certain developmental stages. Rather, H4K20me1 and H3K27me may be pivotal epigenetic marks, although we do not doubt that other histone lysine methyl marks also contain epigenetic information. Finally, we should not forget that Waddington's epigenetic landscape (Waddington, 1957) comprises both peaks and valleys (that is, some regions with high concentrations of epigenetic marks and others that lack them). Thus, histone lysine demethylases could be epigenetic factors themselves if they protect regions from being methylated, and thus from being converted from "epigenetic" valleys to "epigenetic" peaks.

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