Mechanistic and Functional Links Between Histone Methylation and DNA Methylation

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DNA methylation is a common mechanism of epigenetic regulation in eukaryotic organisms ranging from fungi to mammals. Genetic studies in model organisms have demonstrated the involvement of DNA methylation in a variety of biological processes. In mammals, DNA methylation patterns are established and maintained by three DNA methyltransferases: Dnmt3a, Dnmt3b, and Dnmt1. The basis of the specificity of the DNA methylation machinery and how DNA methylation patterns are regulated remain poorly understood. However, accumulating evidence suggests complex interplay between DNA methylation and other epigenetic mechanisms. Of particular interest is histone lysine methylation that has been shown to be tightly linked to DNA methylation in various systems. This chapter highlights the findings of several recent studies that provide insights into the mechanistic and functional interactions between histone methylation and DNA methylation.

I. Introduction

DNA methylation, the covalent addition of a methyl (CH_3) group to the nucleotide cytosine, is an epigenetic modification conserved in most major eukaryotic groups, including many fungi, plants, and animals, although it has been lost in some organisms such as the budding yeast *Saccharomyces cerevisiae* and the nematode worm *Caenorhabditis elegans*.[1] The biological significance and functions of DNA methylation vary among different organisms. In mammals, DNA methylation is essential for embryonic development and

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plays important roles in a variety of biological processes, including gene regulation, suppression of transposable elements, genomic imprinting, and X chromosome inactivation.[2] Aberrant changes in DNA methylation levels and patterns are associated with a number of developmental disorders (such as immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome, Beckwith–Wiedemann syndrome, Prader–Willi syndrome, and Angelman syndrome) and complex diseases (e.g., cancer, immunological abnormalities, and psychiatric disorders).[3–5]

In animal genomes, methylated cytosines are mostly restricted to CG dinucleotides (although non-CG methylation is prevalent in embryonic stem (ES) cells). This is in contrast to plants and some fungi (e.g., *Neurospora crassa*), in which DNA methylation occurs in both symmetric (CG, CHG; H = A, C, or T) and asymmetric (CHH) contexts.[6,7] Overall, 60–80% of all CpG dinucleotides in a mammalian genome are methylated. However, methylated cytosines are not randomly distributed across the genome. Heterochromatin, including centromeric, pericentric, and subtelomeric regions as well as transposable elements, is heavily methylated, and this contributes to the transcriptionally repressed, highly condensed chromatin structure characteristics of these regions. Most coding regions also show high degrees of DNA methylation. In contrast, many CpG islands at 5′ promoters lack DNA methylation. However, in genomic regions where transcription is stably silenced, such as imprinted genes and the inactive X chromosome, promoter-associated CpG islands are methylated, and this methylation is essential for maintaining the silenced state.[8,9]

Three active DNA methyltransferases have been identified in mammals (see Chapters by Željko M. Svedružic; and Frédéric Chédin). Dnmt1 functions primarily as a maintenance methyltransferase that copies the parental strand CpG methylation pattern onto the daughter strand after each round of DNA replication.[10] Dnmt3a and Dnmt3b function as *de novo* methyltransferases that are responsible for establishing DNA methylation patterns during early embryogenesis and gametogenesis.[11,12] Dnmt3a and Dnmt3b also cooperate with Dnmt1 to maintain the levels and patterns of DNA methylation.[13,14]

DNA methylation is mechanistically and functionally linked to other epigenetic mechanisms, including histone modifications. Of particular importance is histone lysine methylation, which has been shown to regulate DNA methylation or act cooperatively with DNA methylation in a variety of biological processes.[15–21] Methylation has been shown to occur at five lysine residues on histone N-terminal tails (H3K4, H3K9, H3K27, H3K36, and H4K20) and one lysine residue within the globular domain of H3 (K79), and these residues can be mono-, di-, or trimethylated. In general, methylation of H3K4, H3K36, and H3K79 correlates with open and transcriptionally active chromatin, whereas methylation of H3K9, H3K27, and H4K20 is associated with condensed and transcriptionally repressive chromatin.[22,23]

Histone lysine methylation is dynamically regulated by protein lysine methyltransferases (PKMTs) and protein lysine demethylases (PKDMs) (see Chapter by L. Aravind *et al.*). All PKMTs, with the exception of the Dot1 family, contain a characteristic SET domain.[24,25] Dot1 family members, which methylate H3K79, contain conserved sequence motifs characteristic of class I methyltransferases such as Dnmts and protein arginine methyltransferases.[25] Two families of PKDMs have been identified. The amine oxidase family has two members, LSD1 (also known as KDM1A or AOF2) and the newly identified KDM1B (also known as LSD2 or AOF1). Both of them use flavin adenine dinucleotide (FAD) as a cofactor, and demethylate mono- and dimethylated, but not trimethylated, lysines. The Jumonji-C (JmjC) domain family, which has multiple members, uses an Fe^{2+}- and 2-oxoglutarate-dependent dioxygenase mechanism, and is able to demethylate mono-, di-, and trimethylated states.[26,27]

In this chapter, I highlight several recent advances in understanding the interplay between histone methylation and DNA methylation, focusing on findings in mammals.

II. An Evolutionarily Conserved Pathway Between H3K9 Methylation and DNA Methylation

How DNA methylation patterns are generated and regulated is poorly understood. Because the Dnmt1 and Dnmt3 families of DNA methyltransferases do not appear to have intrinsic sequence specificity beyond CpG dinucleotides,[28–30] chromatin structure has been suspected to be involved in the regulation of DNA methylation. Consistent with this notion, mutations of genes encoding components of chromatin-remodeling complexes, such as the SNF2 family members, have been shown to alter DNA methylation in plants and animals.[31–33]

The first evidence that histone methylation controls DNA methylation came from studies with the filamentous fungus *N. crassa*.[15] In *N. crassa*, methylated cytosines are extensively distributed within centromeres, telomeres, transposon relics, and repetitive DNA, all of which are products of the genome defense system repeat-induced point mutation (RIP).[34] Unlike in animals and plants, DNA methylation is not essential in *N. crassa*, thereby facilitating genetic studies of DNA methylation in this organism. In a mutagenesis screen, the Selker group identified multiple loci required for DNA methylation, including *dim-2* (defective in methylation 2), which specifies a DNA methyltransferase that is responsible for all known cytosine methylation in *N. crassa*.[35,36] Interestingly, one of the mutants, in which DNA methylation

was entirely eliminated, disrupted a SET domain protein, DIM-5.[15] DIM-5 catalyzes trimethylation of the H3K9 associated with RIP'd DNA.[37] These findings imply that global DNA methylation is dependent on histone H3K9 methylation in *N. crassa*.

Histone H3K9 methylation is also important for some DNA methylation in plants.[16,38] In *Arabidopsis thaliana*, there are three families of DNA methyltransferases. DRM2 (domains rearranged methyltransferase 2), an ortholog of the mammalian Dnmt3 enzymes, appears to carry out all *de novo* methylation.[39] The Dnmt1-like MET1 (methyltransferase 1) primarily maintains CpG methylation,[40,41] while the plant-specific methyltransferase CMT3 (chromomethylase 3), a chromodomain-containing protein, is responsible for the maintenance of CHG and other non-CpG methylation.[42,43] In genetic screens for suppressors of DNA methylation-correlated gene silencing, Jackson et al. and Malagnac et al. isolated mutations in the *KRYPTONITE* (*KYP*) gene (also known as SU(VAR) 3–9 homolog 4 (*SUVH4*)), which produces a histone H3K9 methyltransferase similar to mammalian Suv39h. Loss-of-function *Kyp/suvh4* alleles resembled *CMT3* mutants, showing loss of cytosine methylation primarily at CHG sites and reactivation of endogenous retrotransposon sequences[16,38].

To determine whether a similar connection between histone methylation and DNA methylation is operative in mammals, Lehnertz et al. investigated DNA methylation at various repetitive sequences in *Suv39h1* and *Suv39h2* double null (dn) mouse ES cells.[17] Suv39h1 and Suv39h2 function collaboratively to establish H3K9 trimethylation at pericentric heterochromatin and are required to maintain genome stability.[44] Consistently, the *Suv39h* dn ES cells displayed severe loss of cytosine methylation at pericentric satellite repeats but not at centromeric repeats and other repetitive sequences examined. The authors also showed that H3K9 trimethylation at pericentric heterochromatin is unaltered in *Dnmt1*- or *Dnmt3a/Dnmt3b*-deficient ES cells.[17] These data suggest that DNA methylation at pericentric satellite repeats acts genetically downstream of Suv39h-mediated H3K9 trimethylation. G9a and the closely related GLP/EuHMTase1 form a heteromeric complex and are crucial for H3K9 methylation (mainly mono- and dimethylation) of euchromatin; they also regulate DNA methylation.[45–50] However, G9a appears to recruit Dnmt3a and Dnmt3b independently of its histone methyltransferase activity,[48–50] suggesting a more complex relationship between the histone and DNA methylation systems in mammals.

These studies demonstrated an evolutionarily conserved role of H3K9 methylation in the control of DNA methylation. However, the molecular mechanisms by which histone methylation directs DNA methylation may vary in different organisms. In *N. crassa*, heterochromatin protein 1 (HP1) appears to serve as a bridge between H3K9 methylation and DNA methylation.

HP1 proteins are a highly conserved family involved in heterochromatin formation and gene silencing.[51] These proteins contain a chromodomain, which binds methylated H3K9, and a chromoshadow domain, which interacts with diverse factors.[52] Mutations in the *Neurospora* HP1 gene *hpo* eliminate all detectable DNA methylation, just like null mutations in *dim-2* or *dim-5*.[53] Further, HP1 directly interacts, via its chromoshadow domain, with DIM-2.[54] These data suggest that DNA methylation in *N. crassa* is largely the result of a unidirectional pathway in which DIM-5 methylates histone H3K9 and then the DIM-2/HP1 complex recognizes the resulting trimethyl-H3K9 marks. In *Arabidopsis*, though the only known HP1 ortholog LHP1 interacts with CMT3 *in vitro*, genetic studies revealed that LHP1 is not required for DNA methylation.[16,38,55] However, the chromodomain of CMT3 can interact directly with the histone H3 tail when K9 and K27 are simultaneously methylated.[55] Mammals have three HP1 variants: HP1α, HP1β, and HP1γ. Coimmunoprecipitation experiments show that, in mouse ES cells, Dnmt3b interacts with HP1α and HP1β, but not with HP1γ.[17] In other cell types, Dnmt1, Dnmt3a, and Dnmt3b seem to interact with all three HP1 variants.[56,57] It remains to be determined, however, whether mammalian HP1 proteins are required for DNA methylation.

III. A Role for LSD1 in Coordinating Histone and DNA Methylation?

For a long time, protein lysine methylation was considered to be a permanent modification. This view has changed with the identification of LSD1 and other PKDMs. LSD1 demethylates primarily mono- and dimethyl H3K4, although its substrate specificity appears to be modulated by interacting proteins.[58,59] Recent studies have indicated that the action of LSD1 is not solely directed toward histone proteins. For example, LSD1 has been shown to demethylate p53 at K370 and E2F1 at K185 and regulates the functions of these proteins.[60,61]

Recently, we identified Dnmt1 as a novel substrate for LSD1.[20] We showed that *Lsd1*-deficient mouse ES cells maintained an undifferentiated state when cultured in regular ES medium but underwent cell death upon induction of differentiation and failed to form embryoid bodies and teratomas, a phenotype similar to that of *Dnmt1*- or *Dnmt3a/Dnmt3b*-deficient ES cells.[13,62,63] Indeed, in the absence of LSD1, the cells showed progressive loss of global DNA methylation, suggesting a defect in the maintenance of DNA methylation. Western blot and immunofluorescence analyses showed that the Dnmt1 level was substantially reduced, whereas the levels of Dnmt3a and Dnmt3b were unaltered. The reduction of Dnmt1 was due to enhanced turnover of the

Dnmt1 protein (see Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan). The observation that the deregulation of Dnmt1 in *Lsd1*-deficient cells occurred at the posttranslational level raised the possibility of Dnmt1 being a direct substrate for LSD1. Indeed, metabolic-labeling experiments revealed enhanced methylation of Dnmt1 protein in the absence of LSD1, suggesting that Dnmt1 is subject to LSD1-mediated demethylation *in vivo*. Using a candidate approach, we showed that K1096 (K1094 in human DNMT1), a putative Set7/9 methylation site, can be methylated by Set7/9 and demethylated by LSD1 *in vitro*. However, Dnmt1, which contains more than 120 lysine residues, seems to be methylated at multiple sites, as metabolic-labeling experiments indicated that mutating K1096 in mouse Dnmt1 slightly reduced, but did not abolish, Dnmt1 methylation.[20] A subsequent study indeed showed that Set7/9 can also methylate K142 and reduce the level of Dnmt1 (see Chapter by Shannon R. Morey Kinney and Sriharsa Pradhan).[64] Taken together, these results suggest that Dnmt1 stability is regulated by lysine methylation. LSD1 and Set7/9 (possibly other PKDMs and PKMTs as well), by acting directly on both histones and Dnmt1, may play a role in coordinating histone methylation and DNA methylation (Fig. 1).

Consistent with its role in maintaining DNA methylation, Dnmt1 is ubiquitously expressed in proliferating cells. Although the regulation of Dnmt1 expression is not well understood, multiple factors have been shown to modulate *Dnmt1* transcription. Our finding that the stability of the Dnmt1 protein is regulated by lysine methylation adds additional complexity in the control of Dnmt1 level. Regulation of Dnmt1 at the posttranslational level might be relevant in various biological processes, including embryogenesis and tumorigenesis. In preimplantation embryos, maternally derived Dnmt1o (oocyte

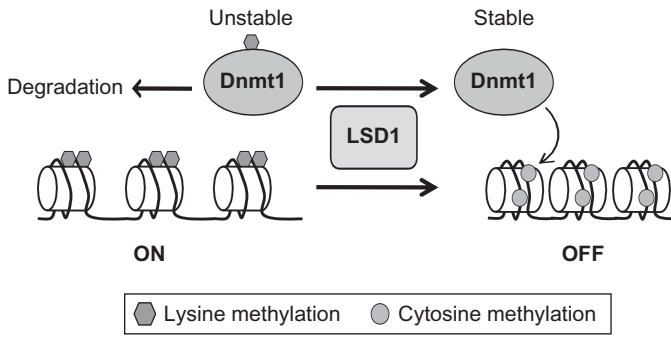

FIG. 1. *A possible role for LSD1 in coordinating histone methylation and DNA methylation.* Methylated Dnmt1 is metabolically unstable. LSD1, by acting directly on both histone H3 and Dnmt1, causes H3K4 demethylation and increased Dnmt1 and DNA methylation, resulting in chromatin condensation and gene silencing.

specific) is the major Dnmt1 variant, which has been shown to be highly stable.[65] Cancer cells often show increased levels of Dnmt1, and enhanced Dnmt1 stability appears to be partly responsible.[66,67]

IV. H3K4 Demethylation and Genomic Imprinting

In contrast to H3K9 methylation, which usually correlates with and promotes DNA methylation, H3K4 methylation seems to protect genomic regions from DNA methylation. Our finding that demethylation of H3K4 is required for the establishment of maternal genomic imprints provides a good example.[21]

Genomic imprinting is an epigenetic process in the germ line that leads to differential modification of the genome in the male and female gametes, resulting in parent-of-origin-specific expression of a small subset of genes ($\sim$100 imprinted genes have been identified in mammals; see Chapter by Jon F. Wilkins and Francisco Úbeda). DNA methylation is believed to be the primary imprinting signal, as all imprinted genes show allele-specific DNA methylation at one or more regulatory regions and deletion of such differentially methylated regions (DMRs) causes loss of imprinting.[68–72] Most methylation imprints are inherited from the mother and are put onto the genome in oocytes by Dnmt3a.[12] Genetic studies demonstrated that *Dnmt3L*, which encodes a protein similar to Dnmt3a and Dnmt3b but without methyltransferase activity, is also essential for germline imprinting.[73,74] Dnmt3L has been shown to interact with Dnmt3a and stimulates its activity.[74–78] A recent study showed that Dnmt3L binds the N-terminal tail of histone H3 and the interaction is inhibited by methylation at H3K4, suggesting that H3K4 methylation may play a role in the establishment of DNA methylation imprints.[79]

In an attempt to test the hypothesis that H3K4 methylation is involved in germline imprinting, we carried out bioinformatic analyses to identify PKMTs and PKDMs that are expressed in germ cells. These analyses, as well as experimental data generated subsequently, indicated that, in adult mice, the LSD1 family member KDM1B (also known as LSD2 or AOF1) is almost exclusively expressed in growing oocytes.[21] Biochemical experiments demonstrated that KDM1B is an active demethylase specific for mono- and dimethyl H3K4.[21,80–82] Targeted disruption of the gene encoding KDM1B (i.e., *Aof1*) showed no effect on mouse development. However, KDM1B-deficient females showed a maternal effect lethal phenotype, similar to mice deficient for *Dnmt3a* or *Dnmt3L*. Indeed, in the absence of KDM1B, growing oocytes showed a substantial increase in H3K4 methylation and failed to set up DNA methylation marks at DMRs of four of seven imprinted genes examined. As a result, embryos derived from KDM1B-deficient oocytes exhibited biallelic expression or biallelic silencing of the affected genes and could not survive

beyond midgestation.[21] Based on these findings, we propose that, during oogenesis, H3K4 methylation needs to be removed to allow *de novo* DNA methylation at imprinted loci (Fig. 2).

Interestingly, phylogenetic analysis indicates that KDM1B is present in mammals and plants but not in insects[83] (see Part II of this volume). This raises the possibility that KDM1B may have evolved specifically for genomic imprinting. However, KDM1B deficiency affects some, but not all, maternal imprints, suggesting that *de novo* DNA methylation at some imprinted loci is either controlled by other H3K4-specific PKDMs (e.g., LSD1) or independent of H3K4 methylation. Although KDM1B does not control the establishment of paternal imprints during spermatogenesis, H3K4 methylation is absent at paternally methylated DMRs at stages preceding the global histone-to-protamine exchange, raising the possibility that removal of H3K4 methylation is also required for setting up imprints in the male germ line.[84] KDM1B overexpression or deficiency had dramatic effects on global H3K4 methylation,

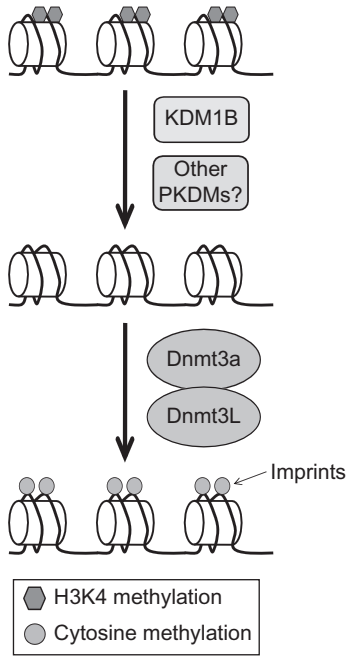

FIG. 2. *A model for the establishment of genomic imprints in female germ cells*. In this model, demethylation of H3K4 by KDM1B (and perhaps other PKDMs) creates docking sites for Dnmt3L, which recruits and/or activates Dnmt3a, and Dnmt3a puts methyl groups on DNA at imprinted loci.

suggesting that KDM1B acts not only on imprinted loci but on many other chromatin regions as well. Yet, KDM1B controls *de novo* DNA methylation at imprinted genes, without affecting global DNA methylation.[21] Therefore, the specificity is probably conferred by the *de novo* DNA methylation machinery. Indeed, the Dnmt3a–Dnmt3L complex has been shown to preferentially methylate DNA sequences with CpG sites 8–10 bp apart.[85] Interestingly, an 8- to 10-bp CpG periodicity is present within DMRs of maternally imprinted genes, although such a structural feature may not be specific to DMRs.[85,86] It is also possible that other histone modifications are required to guide *de novo* DNA methylation at imprinted genes. Further, transcription across imprinting control regions has been implicated in acquisition of maternal imprints.[87]

KDM1B is not highly expressed in most somatic tissues and is not required for mouse development.[21] However, human KDM1B is upregulated in multiple types of cancer, due to amplification of chromosome 6p22, where human *AOF1* is located.[88,89] Indeed, KDM1B has been implicated in gene transcription in human cancer cells.[82] It could be informative to determine whether overexpression of KDM1B plays a role in tumor formation and progression. Genes like *AOF1*, which are not essential for development and physiology but may be critical for cancer cells, are appealing candidate targets for cancer therapy.

V. Concluding Remarks

Over the past two decades, great progress has been made in elucidating the functions of DNA methylation in mammals. Genetic manipulations of DNA methyltransferases have demonstrated the involvement of DNA methylation in a variety of biological processes. However, much less is known about how DNA methylation patterns are generated and regulated. While there is evidence that DNA methylation and other epigenetic mechanisms, such as histone modifications, function collaboratively, the mechanistic links between these systems, in most cases, remain to be determined. In addition, it is largely unknown how DNA methylation and histone modifications work coordinately in the context of development. The finding that KDM1B is required for the establishment of maternal genomic imprints represents a rare example of the intricate interplay between histone methylation and DNA methylation in a developmental process. Moreover, although a large number of histone-modifying enzymes have been identified, investigation of their biological functions has lagged behind. Many of these factors have been implicated in cancer and other diseases. Understanding their roles in normal development and physiology and their links to various diseases will likely open up new avenues for the diagnosis, treatment, and prevention of these diseases (see Part VI of this volume).

In the future, we expect to see more studies that address these issues. Gene targeting in mice, in combination with the ever-advancing "omics" technologies, will continue to be a powerful approach.

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