

CHAPTER 2

Epigenetic Mechanisms of Gene Regulation: Relationships between DNA Methylation, Histone Modification, and Chromatin Structure

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Abstract

DNA methylation is a post-replicative, or epigenetic, modification of the genome that is critical for proper mammalian embryonic development, gene silencing, X chromosome inactivation, and imprinting. Genome-wide DNA methylation patterns are nonrandomly distributed and undergo significant remodeling events during embryogenesis. DNA methylation patterns are also frequently 'remodeled' in tumor cells in a way that directly contributes to tumor suppressor gene inactivation and genomic instability. The mechanisms for the establishment and maintenance of genomic DNA methylation patterns during development and in somatic cells remains a very important and unanswered question in the DNA methylation field. Emerging evidence suggests that protein-protein interactions between components of the DNA methylation machinery (the DNA methyltransferases) and aspects of chromatin structure such as histone tail modifications and chromatin remodeling, directly determine which regions of the genome are to be methylated. By studying these mechanisms in detail we should be able gain insights into how DNA methylation patterns become disrupted in tumor cells and how these defects may be corrected.

Introduction

Methylated DNA refers to DNA strands containing nucleotide bases modified to contain a methyl group ($-CH_3$). Early work in the DNA methylation field centered on the study of the restriction-modification system, a mechanism of bacterial genome protection. In this system, a restriction endonuclease designed to cleave invading viral DNA is coexpressed with a DNA methylase. The DNA methylase methylates the bacterial genome at the same sequence cleaved by the restriction endonuclease, which inhibits cleavage of the host genome and thus selectively destroys the invading DNA sequence.^{1,2} It was not surprising that researchers suspected that similar activities might exist in higher eukaryotes. Beginning in the late 1960s, there were several reports on the purification of an activity from mammalian sources able to produce 5-methylcytosine.³⁻⁵ Although the biochemical properties of 5-methylcytosine in mammalian cells were analyzed extensively through two decades, it was not until 1988 that the first cloning and sequencing of a murine DNA methyltransferase was reported.⁶ This enzyme is now termed DNMT1, and our understanding of the players performing on the stage of mammalian DNA methylation has been growing by leaps and bounds ever since.

From bacteria to humans, DNA methyltransferases are highly conserved during evolution, and are thus regarded as important regulators of a variety of aspects of cellular function.⁷ There are three types of DNA methyltransferases, classified by the nucleotide targeted for modification, N4-methyladenine, N6-methyladenine, and C5-methylcytosine DNA methyltransferases.

Only one type of DNA methyltransferase is known in mammalian cells, 5-methylcytosine DNA methyltransferase, which transfers a methyl group to the 5-position of cytosine within the CpG dinucleotide recognition sequence.⁸ The product of this methylation reaction, 5-methylcytosine, has drawn considerable attention because methylated DNA is believed to be associated with transcriptional regulation and higher order chromatin structure.⁹⁻¹²

In mammals, DNA methylation patterns are not randomly distributed throughout the genome, but rather methylated DNA is localized to discrete regions of the genome enriched in repetitive DNA and transposable elements, imprinted domains, and the inactive X chromosome in females.^{11,13} In these regions, DNA methylation may serve to suppress spurious transcription, transposition, and recombination. Furthermore, DNA methylation patterns are quite dynamic during mammalian development, with genome-wide methylation remodeling events occurring following fertilization and embryo implantation.¹⁴ DNA methylation patterns also change substantially during the process of tumorigenesis and these changes appear to be early events contributing directly to the transformed phenotype.⁹ Tumor cells exhibit global losses of methylation from repetitive sequences and region-specific gains in methylation, primarily within CpG-rich gene regulatory regions known as CpG islands.¹⁵ Promoter region CpG island methylation can silence expression of the associated gene with great efficiency. If the gene is a tumor suppressor gene then the aberrant methylation can provide the cell with a growth advantage as if the sequence had been deleted.^{9,10} Thus, although DNA methylation patterns are generally very stable in somatic cells, they can undergo dramatic changes during embryogenesis and tumorigenesis and these changes have profound effects on cell growth and development.

This chapter will first review what is known about the enzymes that are directly responsible for methylated DNA modification in mammalian cells—the DNA methyltransferases (DNMTs). I will then summarize current knowledge of the proteins known to associate with the DNMTs that may alter their enzymatic activity or nuclear targeting. Finally, I will discuss exciting emerging connections between DNA methylation and histone modifications and chromatin remodeling proteins that may soon provide answers to the perplexing question of how cellular DNA methylation patterns are established during development and maintained in somatic cells. These studies may also shed light on the nature of the defect in the cellular DNA methylation machinery that contributes to cellular transformation.

The Mammalian DNA Methyltransferases (DNMTs)

Five genes encoding DNMTs (including potential DNMT-like genes that may not be enzymatically active) have been identified in mammalian cells, DNMT1, 2, 3A, 3B, and 3L.^{6,16-18} Each gene is designated by the numbers 1, 2, 3, in the order in which they were identified. For the members of DNMT3 family, the additional letters A, B and L were used. The five genes can be divided into three categories, based primarily on function: the maintenance DNA methyltransferase DNMT1, the de novo DNA methyltransferases DNMT3A and DNMT3B, and the DNMT-like proteins DNMT2 and DNMT3L (Fig. 1, Table 1). DNMT1 is referred to as the maintenance methyltransferase due to its preference for hemimethylated DNA (which exists as a by-product of DNA replication),^{19,20} its targeting to replication foci during S-phase,^{21,22} and its interactions with the replication foci-associated proteins proliferating cell nuclear antigen (PCNA),²³ and the retinoblastoma gene product, Rb²⁴ (Fig. 1, Table 4). DNMT1 also has several alternatively spliced sex-specific isoforms which appear to be involved in the establishment of DNA methylation patterns and imprinting in germ cells and the developing embryo²⁵ (Table 2). The de novo DNA methyltransferases, DNMT3A and DNMT3B, have been shown to be essential for the waves of de novo methylation in embryonic cells following implantation.²⁶ These enzymes also mediate de novo methylation of newly integrated parasitic DNA sequences, such as retroviruses, as part of a host cell 'genome defense system'.^{13,26} Like DNMT1, DNMT3B has a number of isoforms resulting from alternative splicing events that are expressed in a tissue-specific fashion and which may alter catalytic activity or DNA binding (Table 2).^{17,27} The DNMT-like proteins DNMT2,¹⁶ and DNMT3L¹⁸ possess all or some of

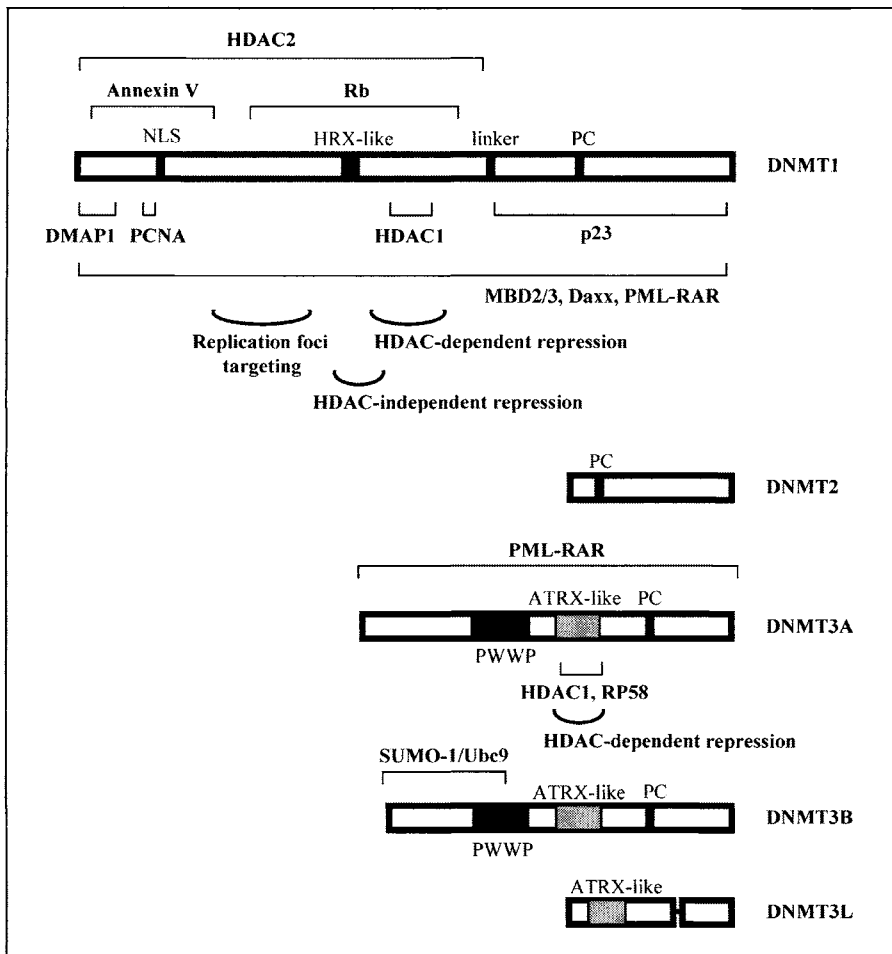


Figure 1. Schematic structures of all known mammalian DNA methyltransferases. Important domains are indicated with boxes and include the nuclear localization sequence (NLS), an HRX-like region, the lysine-glycine repeat region (linker), the catalytic active site proline-cysteine dipeptide (PC), an ATRX-like plant homeodomain region (ATRX-like),¹⁷ and a PWWP motif⁸⁶ Proteins known to interact with each of the DNMTs are shown with brackets to denote the interacting region, where known. A bracket encompassing the entire protein indicates that the interaction domain has not been mapped. Transcriptional repression domains are indicated with rounded brackets. DNMT3L lacks the PC motif.

the highly conserved methyltransferase catalytic motifs, respectively, but have not been shown to display enzymatic activity *in vitro*. Thus their true roles in DNA methylation metabolism remain unclear.

Targeted inactivation of each of the DNA methyltransferase genes in murine embryonic stem (ES) cells has been performed and these studies have provided important information regarding the roles of each DNMT in the establishment and maintenance of genome-wide DNA methylation patterns. The results of all the DNMT knockout studies, summarized in Table 3, indicate that all of the DNMTs that have been shown to possess enzymatic activity *in vitro* are also absolutely essential for proper embryonic development in mice. DNMT1

Table 1. Properties of the mammalian DNA methyltransferases

| DNA Methyl Transferase | Size of Human Protein (Amino acids) | Chromosomal Location | mRNA Expression Profile | Subcellular Localization | Catalytic Activity in Vitro |
|------------------------|-------------------------------------|----------------------|--|--|--|
| DNMT1 | 1616 | 19p13.2 | Placenta, brain, lung, heart, cell cycle dependent ^{87,88} | Nucleoplasm during G1 and G2 phases and replication foci throughout S phase ^{21,22} | Strong (preference for hemimethylated DNA) ¹⁹ |
| DNMT2 | 391 | 10p12-10p14 | Ubiquitous at low levels ^{16,89} | Not determined | Not detected |
| DNMT3A | 912 | 2p23 | Abundant in ES cells, ubiquitous at very low level in embryos and adult tissues, cell cycle independent ^{17,27} | Discrete nuclear foci throughout the cell cycle, however replication foci during late-S phase? ⁴⁴ | Weak (preference for unmethylated DNA) ^{90,91} |
| DNMT3B | 853 | 20q11.2 | Undifferentiated ES cells, embryos, and testis, cell cycle dependent ^{17,27} | Diffuse nuclear distribution in NIH3T3 cells, pericentromeric heterochromatin in ES cells ⁴⁴ | Weak (preference not precisely determined) ⁹² |
| DNMT3L | 387 | 21q22.3 | Testis and embryos ¹⁸ | Not determined | Not determined |

and DNMT3B knockout mice die very early in embryonic development, while DNMT3A knockout mice die soon after birth.^{26,28} Knockout of DNMT3L, which is not likely to encode a functional DNA methyltransferase, resulted in a more subtle phenotype. Homozygous mutant animals of both sexes were viable but sterile and methylation analysis revealed that loss of Dnmt3L resulted in a lack of maternal methylation imprints in homozygous oocytes. Thus it appears that DNMT3L contributes to imprint establishment during oogenesis, but not to genome-wide methylation patterning.²⁹ Although DNMT2 possesses all of motifs believed to be important for catalysis, no enzymatic activity has been detected from DNMT2 and DNMT2-knockout mice appeared completely normal (Table 3).¹⁶

Interaction between DNMTs and Other Proteins

As was mentioned in the introductory remarks, genome-wide DNA methylation is not randomly distributed, yet DNA methyltransferases display little sequence specificity in vitro other than requiring the CpG dinucleotide recognition sequence. Therefore, recent efforts have begun to focus on identifying the protein interaction partners of each of the DNMTs. Emerging evidence suggests that protein-protein interactions dictate which regions of the genome become methylated and which will be protected from methylation.¹¹ This next section will discuss a number of the proteins known to interact with DNMT1, 3A, and 3B (summarized in Fig. 1 and Table 4), and describe how these interactions may mediate DNMT catalytic activity, subnuclear localization, and sequence specificity in vivo.

Table 2. Splice variants of mammalian DNA methyltransferases

| DNA Methyl-transferase Gene | Splice Variants | Tissue Specificity of Expression | Size Difference Compared to the Most 'Typical' Form | Translatable | Catalytic Activity |
|------------------------------------|------------------------|--|---|---|-------------------------------------|
| DNMT1 | DNMT1s | The most typical somatic form (usually referred to as DNMT1) | 1616 aa (human DNMT1) ^{6,87} | Yes | Yes |
| | DNMT1b | Somatic tissues | +16 aa (human), ⁴ -2 aa (mouse), alternatively spliced at exon 4 ^{93,9} | Yes | Yes |
| | DNMT1o | Oocytes | -118 aa (mouse), alternatively spliced at exon 1 ²⁵ | Yes | Yes |
| | DNMT1p | Pachytene spermatocytes and skeletal muscle | -118 aa (mouse), alternatively spliced at exon 1 ^{25,68} | Yes in skeletal muscle, no in spermatocytes | Not determined, but most likely yes |
| DNMT3A | Short form | Undifferentiated ES cells and 10.5 day embryos (usually referred to as DNMT3A) | 912 aa (human DNMT3A), 4.2-kb in murine cells ¹⁷ | Yes | Yes |
| | Long form | Most adult tissues and differentiated embryos, not in ES cells | 9.5-kb as murine mRNA (possibly containing large 5' UTR?) ¹⁷ | Not determined | Not determined |
| DNMT3B | DNMT3B1 | Undifferentiated ES cells, embryos, and testis | 853 aa (human DNMT3B) | Yes | Yes |
| | DNMT3B2 | Undifferentiated ES cells, embryos, and testis | -20 aa (human and mouse) ²⁶ | Yes | Yes |
| | DNMT3B3 | Undifferentiated ES cells, embryos, and testis | -63 aa (human and mouse) ²⁶ | Yes | No |
| | DNMT3B4 | Testis | -109 aa, alternatively spliced at C-terminal region (human) ²⁷ | Not determined | Not determined |
| | DNMT3B5 | Testis | -41 aa, alternatively spliced at C-terminal region (human) ²⁷ | Not determined | Not determined |

Table 3. Effects of targeted disruption of DNA methyltransferase genes in mice

| DNA Methyltransferase | Homozygous ES Cells | Heterozygous Knockout Mice | Homozygous Knockout Mice |
|-----------------------|---|-----------------------------------|---|
| DNMT1 | Viable and normal morphology, 70% decrease in total 5-methylcytosine. | Indistinguishable from wild type. | Failed to develop beyond mid-gestation, embryonic lethality. ²⁸ |
| DNMT1 α | Viable, characteristics not described in detail. | Normal | Homozygous mutant males showed normal fertility. Homozygous mutant females were infertile. Heterozygous offspring of homozygous females showed demethylation at certain imprinted loci, but not over the whole genome. ⁹⁵ |
| DNMT2 | Viable and normal. | Normal | No significant phenotype. ¹⁶ |
| DNMT3A | Viable and normal undifferentiated morphology. The de novo methylation activity on proviral DNA was normal. Centromeric-minor satellite DNA repeats were normally methylated. | Normal and fertile. | Appeared normal at birth, showed undergrowth at 18 days and died by 4 weeks of age. Retroviral DNA was methylated at normal levels. ²⁶ |
| DNMT3B | Viable and normal undifferentiated morphology. The de novo methylation activity on proviral DNA was normal. Centromeric-minor satellite DNA repeats were substantially demethylated. | Normal and fertile. | No viable homozygous mice were born. Retroviral DNA was slightly undermethylated. ²⁶ |
| DNMT3A and DNMT3B | Double mutant ES cells completely lacked de novo methylation activity on proviral DNA. Centromeric-minor satellite DNA repeats were demethylated to the same level as DNMT3B-/- ES cells. | Not reported. | Double homozygous embryos showed smaller size at E8.5 and died before E11.5. Retroviral DNA was highly undermethylated. ²⁶ |
| DNMT3L | Viable, characteristics not described in detail. | Normal and fertile. | Both sexes born normal but sterile. Adult testes had severe hypogonadism. Females showed a maternal-effect lethal in that heterozygous progeny of homozygous females died before midgestation. Maternal methylation imprints were markedly disrupted, while genome-wide methylation patterns were normal. |

Table 4. Proteins interacting with mammalian DNA methyltransferases

| DNA Methyl-transferase | Interacting Protein | Function of Inter-acting Protein | Possible Role in Vivo |
|------------------------|---------------------|--|--|
| DNMT1 | HDAC1/2 | Histone deacetylase | Modification of chromatin by histone deacetylation, resulting in chromosome condensation, targeting DNA methylation? ^{24,42,46} |
| | Rb | Tumor suppressor, Cell-cycle regulation | Sequester DNMT1 in non-dividing cell, target or modulate DNMT activity at replication foci? ^{24,37} |
| | DMAP1 | Co-repressor | Recruiting other repressors, transcriptional repression. ⁴⁶ |
| | PML-RAR | Oncogenic transcription factor | DNA-binding and interaction with other transcriptional co-regulators, targeting methylation. ⁶² |
| | MBD2/3 | Methyl-CpG binding proteins | Transcriptional repression in methylated regions, possible targeting of DNMT1 to hemimethylated DNA at replication foci? ⁶⁵ |
| | PCNA | "Sliding platform" that can mediate the interaction of proteins with DNA, essential for processivity of DNA polymerase | Targeting DNMT1 to replication foci. ²³ |
| | p23 | Subunit of a progesterone receptor complex | Proper protein folding, regulation of DNMT1 catalytic activity? ⁹⁶ |
| | Daxx | Transcriptional repressor? | Connection between DNMT1 and other transcription factors, mediate PML – DNMT1 interaction? ⁹⁷ |
| DNMT1o | Annexin V | Ca-2+ dependent phospholipid-binding protein | Cytosol-nuclear translocation of Dnmt1o during oogenesis via membrane trafficking? ⁶⁷ |
| DNMT3A | HDAC1 | Histone deacetylase | Modification of chromatin by histone deacetylation, targeting DNA methylation? ^{43,44} |
| | RP58 | Transcription factor | Sequence-specific DNA binding, targeting repression, maybe methylation as well? ⁴³ |
| | PML-RAR | Oncogenic transcription factor | DNA-binding and interaction with other transcriptional co-regulators, targeting methylation. ⁶² |
| DNMT3B | HDAC1 | Histone deacetylase | Modification of chromatin by histone deacetylation, targeting DNA methylation? ⁴⁴ |
| | SUMO-1/Ubc9 | Sumo ligase | Modification of protein by sumoylation, altered localization or enzymatic activity? ⁹⁸ |

DNA Methylation and DNA Replication

PCNA

PCNA, or the polymerase processivity factor, is an essential protein in DNA replication. Its heterotrimeric ring-shaped structure allows PCNA to encircle double-stranded DNA and provide a platform for the assembly of other replication-associated proteins.³⁰ PCNA has been shown to bind to a number of other cellular proteins involved in DNA replication, mismatch repair, and cell cycle regulation.³¹ One of these interacting factors is DNMT1 (Fig. 1).²³ DNMT1 was shown to bind to and colocalize with PCNA at early S-phase replication foci. PCNA binding to DNMT1 did not affect its methyltransferase activity, suggesting that PCNA does not regulate DNMT1 activity directly. Rather, DNMT1 appears to be recruited by PCNA to foci of newly replicated DNA to allow for remethylation of hemimethylated DNA. This result is consistent with the traditional model of DNMT1 as a maintenance methyltransferase of newly replicated DNA (Table 4).²³

Rb

The retinoblastoma tumor suppressor protein (Rb) controls cell growth by regulating the expression of genes that promote cell cycle progression. Hypophosphorylated Rb binds to the transcription factor E2F and represses its activation function by recruiting histone deacetylases (HDACs) and histone methylases (HMTs).³²⁻³⁴ When a cell is ready to divide, Rb is phosphorylated, dissociates from E2F, and transcriptional activation occurs.³⁵ Rb itself, or other members of the Rb regulatory pathway, are mutated in nearly all tumor cells, emphasizing the importance of Rb-mediated growth control in normal cells.³⁶ DNMT1 was found to copurify with Rb and E2F1 and interact directly with Rb (Fig. 1). DNMT1 could enhance Rb-mediated repression specifically at E2F-responsive promoters and the repression was partially HDAC-dependent but was independent of the DNA methyltransferase activity of DNMT1.²⁴ The amino terminal region of DNMT1 containing the cysteine-rich domain was found to interact with the A/B pocket domain of Rb²⁴ and later the B/C pocket region as well.³⁷ Interestingly, DNA methyltransferase activity was strongly inhibited by binding to Rb and this effect appeared to be mediated by interfering with the ability of DNMT1 to bind to DNA. This suggested that Rb may modulate DNMT1 activity in vivo, which was confirmed when Rb was over expressed in cells and a genome-wide reduction in 5-methylcytosine was observed.³⁷ Rb, as well as DNMT1 and PCNA, colocalizes with early S-phase perinucleolar foci that correspond to sites of active DNA synthesis.³⁸ Therefore, the potential roles of the Rb-DNMT1 interaction may be to sequester and repress DNMT1 enzymatic activity in non-dividing cells,¹¹ target DNMT1 to replication foci, regulate the association of DNMT1 with PCNA, or reduce or inhibit the catalytic activity of DNMT1 specifically at early S-phase replication foci (Table 4). Interestingly, cancer-specific mutations in the A/B pocket region of Rb were shown to inhibit binding to DNMT1. Therefore mutations in Rb, or the Rb pathway, may directly lead to unscheduled or aberrant DNA methylation events in nondividing cells which would then be copied and spread with each round of cell division.²⁴

Links between DNA Methylation and Histone Modification

HDAC1 and HDAC2

DNA methyltransferase and histone deacetylase (HDAC) are believed to operate along the same mechanistic pathway to silence gene expression. DNA methyltransferases establish and maintain 5-methylcytosines in the context of chromatin and methyl-CpG binding proteins of the MBD family, such as MeCP2, recognize and bind methylated DNA and recruit the corepressor/HDAC protein complex.¹² The catalytic unit HDAC can remove acetyl groups from the core histone tails, leading to assembly of tight-packed chromatin and rendering a promoter inaccessible to the transcription machinery by increasing the affinity of histones for DNA. It

has long been known that transcriptionally inactive regions are hypermethylated and enriched in hypoacetylated histones. Thus the finding that proteins which bind specifically to methylated DNA interact with and recruit HDACs tied these seemingly unrelated observations together.³⁹⁻⁴¹ However, this still leaves open the question of how the region was targeted for DNA methylation to begin with. Recent results from several laboratories have revealed that DNA methylation and histone acetylation may be even more tightly linked than first thought because DNA methyltransferases and histone deacetylases directly interact.^{24,42-44} In fact HDACs have now been found to interact with all of the catalytically active DNMTs and DNA methylation and histone deacetylation act synergistically to repress transcription.⁴⁵

Initial studies with DNMT1 indicated that the amino terminal regulatory domain could act as a transcriptional repressor when fused to a heterologous DNA binding domain.⁴² DNMT1-mediated transcriptional repression was shown to be comprised of both HDAC-dependent and HDAC-independent components. The HDAC-dependent component was defined using the HDAC inhibitor trichostatin A (TSA), which relieves a substantial amount of the DNMT1-mediated repression.^{24,42} DNMT1 was shown to bind HDAC1 via a transcriptional repression region adjacent to the HRX-homology domain,⁴² and a direct interaction between the DNMT1 amino terminal regulatory domain and HDAC2 has also been demonstrated.⁴⁶

Both DNMT3A and DNMT3B are also capable of conferring transcriptional repression when fused to heterologous DNA binding domains via both HDAC-dependent and HDAC-independent mechanisms. Yeast two-hybrid studies have shown that DNMT3A and DNMT3B interact with HDAC1 through the PHD region (Fig. 1, Table 4),^{43,44} which is not present in DNMT1. Therefore, it is possible that the repressive capabilities of DNMT1 and the DNMT3s may be caused by distinct protein-protein interactions, with histone deacetylase as a common mediator.

The functional significance of the DNMT-HDAC interaction remains unclear, although possible roles will be suggested here and in the DNA methylation and chromatin remodeling section to follow. Data from a number of sources indicates that transcriptional silencing occurs before DNA methylation and this transcriptional shutdown may be mediated by histone modifications (deacetylation and methylation) and chromatin remodeling events. For example, silencing of transcription from the X chromosome destined to be inactivated in female cells and histone deacetylation occurs before DNA methylation.⁴⁷ Silencing of transcription from newly introduced retroviral sequences also occurs before de novo methylation and can occur in the complete absence of DNMT3A and DNMT3B, the enzymes thought to mediate retroviral DNA methylation.⁴⁸ A useful analogy may be that histone tail modifications as well as chromatin remodeling, may 'close the door' on transcription while DNA methylation is the 'deadbolt lock' which ensures that the door remains closed. This model implies that histone deacetylation sets the stage, or targets, DNA methylation to a particular region by establishing a particular chromatin configuration or signature recognized by other DNMT-associated proteins or the DNMT itself and which promotes DNA methylation of the region. Recent dramatic results with studies of histone methylation strongly support this notion.

DNA Methylation and Histone Methylation

Histone tail acetylation has received much attention over the last several years as a key mediator of chromatin structure and transcriptional regulation. The histone tails can also be methylated on select residues, such as lysine and arginine, and depending on the amino acid and the histone modified, may exert a stimulatory or inhibitory effect on transcription.^{49,50} Methylation of lysine 9 on histone H3 (H3K9) is associated with transcriptionally silent, heterochromatic regions of the genome.⁵¹ Interestingly, H3K9 methylation also occurs before DNA methylation during X chromosome inactivation.⁵² More direct evidence of a connection between DNA and histone methylation comes from two recent fascinating studies in *Neurospora* and *Arabidopsis*. In *Neurospora*, mutation of a H3K9 methyltransferase gene called *dim-5*

eliminated all detectable cytosine methylation in the *Neurospora* genome.⁵³ In *Arabidopsis*, mutation of a gene homologous to *dim-5* termed *kryptonite*, results in substantial losses of methylation from the CpNpGp sequence, which, unlike mammals, is frequently methylated in plants.⁵⁴ In the latter case, it was demonstrated that the plant DNA methyltransferase responsible for CpNpGp methylation, chromomethylase 3, is targeted to DNA via interaction with the methylated lysine binding protein HP1.⁵⁵ Although DNA methylation is dispensable in *Neurospora* and mammals do not appear to have chromomethylases, a protein homologous to *dim-5* and KRYPTONITE exists in mammals (SUV39H1) and is currently the subject of intense study.^{56,57} We should therefore soon find out if histone methylation is a critical mediator of DNA methylation in mammals, but regardless of the existence of a homologous system in mammals, this example further reinforces the notion that chromatin modifications may set the stage for DNA methylation.

DNMTs As Transcriptional Corepressors

As we described above, DNMTs are associated with transcriptional repression in an HDAC-dependent manner. This fact, however, is not the only aspect of gene silencing mediated by DNA methyltransferases because they are also involved in transcriptional repression in an HDAC-independent (or TSA insensitive) manner. In fact all catalytically active DNMTs possess transcriptional corepression activity that is independent of histone deacetylase activity.^{42,44} Interestingly, the domain responsible for the HDAC-independent repression is different between DNMT1 and the DNMT3s. The region of DNMT1 mediating this effect comprises the cysteine-rich / HRX homology domain (Fig. 1).⁴² In contrast, an amino terminal region, which does not include the cysteine-rich domain, is responsible for the HDAC-independent repression capability of DNMT3A and DNMT3B (Fig. 1).⁴⁴ Although the protein-protein interactions responsible for the HDAC-independent repression are not well characterized, it is clear that mammalian DNMTs are multi-functional proteins capable of modulating transcription. In the following section, we review the DNMT-associated proteins that may contribute to the ability of the DNMTs to repress transcription and target methylation (Fig. 1, Table 4).

PML-RAR

PML-RAR is an oncogenic fusion protein resulting from the reciprocal translocation of the promyelocytic leukemia (PML) gene on chromosome 15 and the retinoic acid receptor α gene (RAR) on chromosome 17, and gives rise to acute promyelocytic leukemia (APL). Although the function of PML in normal cells remains unclear, it appears to be a critical component of discrete nuclear structures referred to as PML-oncogenic domains (PODs, ND10, or nuclear bodies).⁵⁸⁻⁶¹ The PML-RAR fusion protein disrupts the PODs, however they can be restored by treating cells with retinoic acid (RA) since PML-RAR retains both the DNA and ligand binding domains of RAR α . RA treatment also results in differentiation of APL cells, indicating that PODs have an important role in promyelocyte differentiation.^{58,62} A recent study showed that both DNMT1 and DNMT3A interacted with PML-RAR (Fig. 1, Table 4) and recruited the DNMTs to a PML-RAR target gene promoter resulting in transcriptional silencing and de novo methylation.⁶² In the absence of retinoic acid, conditions where PML-RAR acts as a transcriptional repressor, HDAC-dependent transcriptional silencing of an RAR target gene occurred early on, and was then followed by promoter region de novo methylation.⁶² This work represents the first example of protein-protein interactions being able to target DNA methylation to particular genomic regions and again stresses the intimate relationships between DNA methylation and histone deacetylation.

DMAPI

Yeast two hybrid screens using the amino terminal regulatory region of DNMT1 as bait identified a novel factor, DNMT1 associated protein (DMAP) 1, that interacts directly with

the first 120 amino acids of DNMT1 (Fig. 1, Table 4).⁴⁶ It was assumed that DMAP1 also acted as a transcriptional repressor and subsequent two hybrid screens with DMAP1 as bait identified the potent transcriptional repressor, TSG101, as a binding partner of DMAP1. The interaction of DMAP1 and DNMT1 may be responsible for the HDAC-independent component of DNMT1-mediated transcriptional repression. DNMT1 and DMAP1 colocalized at replication foci throughout S-phase, while DNMT1 and HDAC2 colocalized only at late S-phase replication foci. These results led to the suggestion that DNMT1, DMAP1, and HDAC2 may participate in the restoration of heterochromatin structure following DNA replication. DNMT1 and DMAP1 would act to restore DNA methylation patterns following replication throughout S phase while the recruitment of HDAC2 to late replication foci, when hypoacetylated, transcriptionally silenced regions are usually replicated, may allow for rapid deacetylation of newly deposited histones.⁴⁶

RP58

DNMT3A and DNMT3B were recently shown to interact directly with a protein called RP58 via the PHD region within the amino terminal regulatory domain (Fig. 1).⁴³ This region of DNMT3A and DNMT3B also mediates the interaction with HDAC1. RP58 is a sequence-specific zinc finger DNA binding protein and transcriptional repressor associated with heterochromatin.^{63,64} The capacity of RP58 to repress transcription was enhanced by coexpression of DNMT3A, however, this cooperative effect did not require a catalytically active form of DNMT3A.⁴³ This suggests that DNMT3A acts as a structural component in the RP58-mediated repression pathway. Nuclear localization studies using a DNMT3A fragment lacking the catalytic domain also support this notion. The isolated amino terminal domain of DNMT3A colocalized with heterochromatin-associated proteins like HP1 α and methyl-CpG binding proteins like MeCP2.⁴⁴ The colocalization of DNMT3A with other known heterochromatin-associated proteins therefore suggests that DNMT3A may be an important component of hypermethylated, pericentromeric heterochromatin.

MBD2 and MBD3

An interaction between DNMT1 and the methyl-CpG binding proteins MBD2 and MBD3 has been reported (Fig. 1). DNMT1 coimmunoprecipitated with MBD2 and MBD3 and MBD2/MBD3 demonstrated colocalization with DNMT1 at late S-phase replication foci. Furthermore, the MBD2/MBD3 complex exhibited binding affinity for both hemimethylated and fully methylated DNA and repressed transcription in an HDAC-dependent fashion.⁶⁵ These interactions may have roles in directing DNMT1 to hemimethylated sequences following DNA replication, silencing of genes during S-phase, or deacetylation of newly deposited histones in a manner akin to the previously described DNMT1-DMAP1-HDAC2 complex (Table 4).

Daxx

The precise function of Daxx remains unclear. Roles for Daxx in apoptosis have been proposed,⁶⁶ but more recent data indicates that Daxx may be an HDAC-dependent transcriptional repressor.⁶⁰ Daxx has been found to interact with a rather diverse group of proteins in yeast two hybrid screens, including Fas, CENP-C, Pax-3, PML, and DNMT1 (Fig. 1).⁶⁶ The latter two are of interest here, especially in light of the connection between PML, RAR and DNMT1 described earlier. Daxx interacts with and colocalizes with PML in the PODs and the interaction with PML, but not the PML-RAR fusion, inhibits the repression function of Daxx. In cells lacking PML, Daxx resides in regions of condensed chromatin, consistent with a role in transcriptional repression.⁶⁰ The previous study describing the interaction between DNMT1 and PML-RAR⁶² did not determine if the interaction between the two proteins was direct or indirect. Thus one potential function of the Daxx may be as a bridge between DNMT1 and PML-RAR (Table 4).

Annexin V

The amino terminal regulatory domain of DNMT1 interacts directly with annexin V (Fig. 1). It was also reported that this interaction was enhanced by calcium.⁶⁷ Annexin V is localized mainly in the cytosol, but has also been detected in the plasma membrane and the nucleus. In oocytes and four-cell embryos, both annexin V and DNMT1 α , the oocyte specific isoform of DNMT1 (Table 2), colocalize in the cytoplasm.⁶⁸ Annexin V exhibits calcium-dependent binding to acidic phospholipids in the cytosol, suggesting that this protein participates in membrane-related transactions (such as membrane organization, exocytosis, and endocytosis).⁶⁹ Although the function of annexin V is poorly understood, the colocalization of annexin V and DNMT1 α may help to explain the unique cytoplasmic-nuclear translocation events of DNMT1 α during oogenesis and annexin V may somehow be involved in anchoring DNMT1 α in the cytoplasm at this stage (Table 4).

DNA Methylation and Chromatin Remodeling

Three highly significant studies over the last few years have revealed additional links between DNA methylation and chromatin structure other than the previously described interactions between DNMTs and HDACs and histone tail modifications. While covalent modification of the core histone tails by acetylation, methylation, and phosphorylation is a major method of regulating chromatin structure, another mechanism involves ATP-dependent chromatin remodeling machines. These remodeling enzymes, or ATPases, use the energy derived from ATP hydrolysis to directly mobilize or slide nucleosomes on the DNA to permit greater access of transcription factors to DNA and therefore promote activation. Alternatively, they may reorganize nucleosomes into a more regularly spaced, closely packed format which is inhibitory to transcription.⁷⁰⁻⁷² The ATP-dependent chromatin remodeling enzymes that perform this reaction are members of the SNF2 family, so named for the first such protein identified in yeast (*sucrose nonfermenter*). The SNF2 superfamily can be divided into three subfamilies, SNF2-like, ISWI, and CHD, based on the presence of several conserved motifs.^{72,73} SNF2 proteins are involved in transcription, DNA repair, recombination, and chromatin remodeling and have been shown to be able to assemble regularly spaced nucleosomal arrays on DNA *in vitro*, promote ATP-dependent disruption of a periodic nucleosomal array, stimulate factor binding, and alter nucleosome spacing.⁷⁰⁻⁷² I will next review the connections between DNA methylation and chromatin remodeling and then discuss how these processes may be connected mechanistically.

The first connection between DNA methylation and chromatin remodeling came from studies in *Arabidopsis*. Mutation of a gene called *DDM1* (decrease in DNA methylation) yielded plants with numerous growth defects and a profound loss of genomic 5-methylcytosine. The growth defects and losses of DNA methylation became progressively greater with increasing generations of inbreeding.⁷⁴ Rather than a DNA methyltransferase, DDM1 is a member of the SNF2 family of ATPases. Further evidence of a connection between DNA methylation and chromatin remodeling comes from studies of a human protein called ATRX. The *ATRX* gene is mutated in a human genetic disease called ATR-X syndrome (α -thalassemia, mental retardation, X-linked).⁷⁵ ATRX is a member of the CHD subfamily of ATPases and has been shown to associate with transcriptionally inactive heterochromatin and due to its structure, may have a role in chromatin remodeling. ATRX patients also demonstrated DNA methylation defects, although they were far more subtle than the DDM1 mutation, and included both aberrant hypomethylation and hypermethylation events occurring at several repetitive elements in the genome.⁷⁶ Lastly, a recent study made use of transgenic mice to produce a knockout of the murine homolog of DDM1 termed Lsh (lymphoid specific helicase, Hells, PASG).⁷⁷⁻⁷⁹ Lsh-deficient mice died soon after birth with renal lesions and exhibited 50-60% reductions in genomic 5-methylcytosine levels that affected repetitive elements, single copy genes, and genomic imprinting control regions.⁷⁹ Thus inactivation of three putative ATP-dependent chromatin remodeling enzymes in plants and mammals has yielded defects in DNA methylation ranging from subtle to profound.

The three studies just described, especially the Lsh knockout study, provide compelling evidence for a connection between DNA methylation and chromatin remodeling. They also suggest, like the previously mentioned studies of histone methylation, that chromatin remodeling events can determine cellular DNA methylation patterns. This implies that chromatin remodeling takes place before DNA methylation and the DDM1, ATRX, and Lsh results indicated that both *de novo* and maintenance methylation, or both, could be affected. How then might histone tail modifications (such as acetylation), chromatin remodeling, and DNA methylation be linked? There can be little doubt that the linkage between DNA methyltransferases and histone deacetylases is direct and that this linkage is common to all DNMTs. It remains unclear if the ATPase(s) required for proper DNA methylation patterning is directly associated with a particular DNMT, or if the remodeling enzyme exerts its effects transiently, before the DNMT can be directed to its target DNA sites, then departs. Whether the association of the remodeling enzyme and DNMT is direct or indirect, accumulating evidence strongly suggests that modification of chromatin structure, or the establishment of a particular chromatin configuration or 'signature', must be created for the DNA methyltransferases to be directed to regions of the genome to be methylated. This system could be operational during DNA replication, when DNMT1 must access newly replicated DNA to ensure methylation patterns are faithfully copied. It could also be operational during development when genome-wide DNA methylation patterns are remodeled to first erase parental DNA methylation patterns, and then *de novo* methylation events establish the proper DNA methylation pattern of the developing organism.

Studies with the ISWI chromatin remodeling enzyme have shown that prior histone acetylation inhibits the ability of ISWI to bind to and remodel nucleosomes.⁸⁰ If this is a property of other remodeling enzymes like ATRX or Lsh, then the interaction of DNMTs with HDACs would be highly logical. The HDAC would first deacetylate the region to be methylated, the remodeling enzyme would create a chromatin configuration optimal for the DNMT, then the DNMT would access and methylate DNA (Fig. 2). Alternatively, the region destined for DNA methylation may first have to be methylated on histones before the remodeling enzyme and the DNMT can carry out their functions. These models will likely become testable in the very near future and may finally provide the answer to the complex and perplexing question of how DNA methylation patterns are established and maintained. The next important question to be answered will be then be how the region to be methylated is first targeted for transcriptional silencing and histone deacetylation (or methylation). This process may also involve chromatin remodeling⁸¹ or may be the default setting in the absence of transcription or promoter-specific transcription factors.

Answers to these questions should also provide essential insights into the cause of the DNA methylation defects observed in cancer cells. DNA methylation patterns, chromatin structure, and histone tail modifications may all become disrupted in tumor cells.⁸²⁻⁸⁴ ATP-dependent chromatin remodeling enzymes themselves have been found to be mutated in genetic diseases and cancer.^{84,85} Assuming that DNA methylation, histone acetylation, and chromatin remodeling are directly linked as described above, it will be important to determine the relative contribution of aberrations in each of these pathways to the formation of aberrant DNA methylation patterns. If normal histone tail modifications and chromatin structure become disrupted in tumor cells, then when do these aberrations occur during the transformation process? Do they precede the DNA methylation defects? If so then DNA methylation abnormalities may be the end result of other regulatory problems and be most visible to researchers due to the extremely efficient and heritable gene silencing afforded by DNA methylation. If histone acetylation defects occur very early during tumorigenesis for example, then drugs which affect the activity of histone deacetylases or histone acetyltransferases (HATs) may be preventative and the subsequent DNA methylation changes might not occur (Fig. 2). Later stages of tumorigenesis, in which aberrations in multiple epigenetic control mechanisms have already occurred, including DNA methylation changes, may require combination therapies to reverse not only

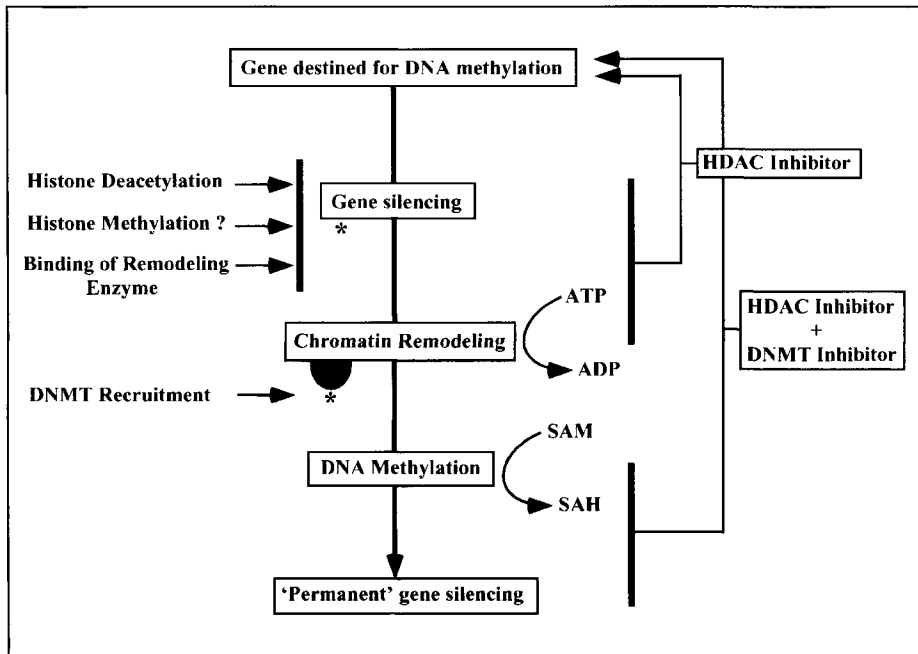


Figure 2. Possible pathway for targeting DNA methylation to specific regions of the genome. A gene destined for long term silencing may first be shut off by histone deacetylation and histone methylation. This then creates an environment favorable for the binding of a chromatin remodeling enzyme (denoted with an *). The chromatin remodeling enzyme binds and repositions or slides nucleosomes in such a way as to directly promote the binding of a DNA methyltransferase or DNMT-associated protein. Alternatively, the remodeling enzyme creates a 'signature' (denoted by black half circle and *), departs, then a DNMT complex binds and methylates DNA. This results in stable long term silencing of the gene. The DNA methylation also recruits methyl-CpG binding proteins and their associated corepressor activities (including histone deacetylase), to further reinforce transcriptional silence and chromatin compaction (not shown). In tumor cells, transcriptional shutdown of key growth regulatory genes may occur before DNA methylation, as is the case in several normal cellular de novo methylation processes. HDAC inhibitors (and possibly chromatin remodeling enzyme and histone methylase inhibitors) can reverse aberrant gene silencing if applied before DNA methylation occurs. Once the region is methylated, an HDAC inhibitor and a DNA methylation inhibitor must be applied to reverse the aberrant gene silencing.

the aberrant histone modifications or chromatin structures, but also the DNA methylation. This is because of the 'permanence' of DNA methylation and its apparent dominance over histone modifications, once established.⁴⁵ Thus it will be critical not only to develop novel DNA methyltransferase inhibitors, but also inhibitors of HDACs, HATs, histone methyltransferases, and ATPases. With these inhibitors in hand it may then be possible to design novel treatment regimens to reverse the epigenetic defects at various stages in the tumorigenesis process.

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