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Interplay between DNA methylation, histone modification and chromatin remodeling in stem cells and during development

KOHTA IKEGAMI[1,#], JUN OHGANE[1], SATOSHI TANAKA[1], SHINTARO YAGI[1], and KUNIO SHIOTA*[,1,2]

[1]*Laboratory of Cellular Biochemistry, Animal Resource Sciences / Veterinary Medical Sciences, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Tokyo and* [2]*Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba-city, Japan*

ABSTRACT Genes constitute only a small proportion of the mammalian genome, the majority of which is composed of non-genic repetitive elements including interspersed repeats and satellites. A unique feature of the mammalian genome is that there are numerous tissue-dependent, differentially methylated regions (T-DMRs) in the non-repetitive sequences, which include genes and their regulatory elements. The epigenetic status of T-DMRs varies from that of repetitive elements and constitutes the DNA methylation profile genome-wide. Since the DNA methylation profile is specific to each cell and tissue type, much like a fingerprint, it can be used as a means of identification. The formation of DNA methylation profiles is the basis for cell differentiation and development in mammals. The epigenetic status of each T-DMR is regulated by the interplay between DNA methyltransferases, histone modification enzymes, histone subtypes, non-histone nuclear proteins and non-coding RNAs. In this review, we will discuss how these epigenetic factors cooperate to establish cell- and tissue-specific DNA methylation profiles.

KEY WORDS: *epigenetics, DNA methylation, T-DMR, histone modification, chromatin remodeling*

Epigenetic systems in mammalian development

In unicellular organisms, each individual cell uses almost all of the genomic information and displays an essentially identical phenotype. In contrast, in mammals there are at least a few hundred different cell types based on a variety of physiological and morphological criteria. All of these cell types are derived from a single fertilized egg. The differentiation of each cell type is achieved without changes in DNA sequence, but through the coordinated utilization of subsets of genes. In order to achieve the proper temporal and spatial regulation of these genes throughout development, a set of epigenetic mechanisms are employed, which includes histone modifications and DNA methylation (Shiota, 2004; Lieb *et al.*, 2006).

In mammals, DNA methylation occurs through DNA methyltransferases (Dnmts) that operate within 5'-CG-3' dinucleotide (CpG: C followed by G). 5-Methylcytosine (5-MeC) is the only chemical modification of mammalian genomic DNA. The status of methylated CpGs is maintained after DNA replication by Dnmts and therefore is heritable through cellular generations (Bird, 2002). 5-MeC is found in most eukaryotic DNA including fungi, plants and vertebrates (Bird, 2002). The prototype Dnmt is found in unicellular organisms such as *Escherichia coli*. The Dnmt genes are conserved among most of eukaryotes, however 5-MeC is absent or rare in the yeast species (*Saccharomyces cerevisiae*, *Saccharomyces pombe*), fly (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*, *Pristionchus pacificus*) due to

Abbreviations used in this paper: 5-MeC, 5-methylcytosine; Dnmt, DNA methyltransferase; E, embryonic day; ES, embryonic stem cell; nt, nucleotide; T-DMR, tissue-dependent differentially methylated regions.

***Address correspondence to:** Dr. Kunio Shiota. Laboratory of Cellular Biochemistry, Animal Resource Sciences / Veterinary Medical Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81-3-5841-8189. e-mail: ashiota@mail.ecc.u-tokyo.ac.jp

[#] **Note:** Present address is Department of Biology, CB# 3280, 405 Fordham Hall, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280, USA.

Note: We use NCBI official symbols for protein or gene names unless otherwise noted (http://www.ncbi.nlm.nih.gov/sites/entrez).

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deletion of the gene or mutations that severely affect the enzymatic activity (Goll and Bestor, 2005). Therefore, the adoption and/or maintenance of the DNA methylation system may be a key molecular event in evolution for species divergence and the emergence of vertebrates.

Histone modifications are another form of epigenetic regulation, which refers to post-translational modifications to the N-terminal tails of the histone. Thus far, over 60 different residues on the core histones (H2A, H2B, H3, and H4) have been reported to be modified. The modifications include acetylation, methylation, phosporylation, ubiquitination, sumoylation, ADP ribosylation, deimination, and proline isomerization (Kouzaridez, 2007). Several histone modifications serve to coordinate the set of 5-MeC. These epigenetic marks are generally associated with chromatin condensation, which plays a crucial role in silencing genes, stabilizing chromosomal structure, and suppressing the mobility of retrotransposons.

Cellular differentiation involves massive alterations of the genome-wide epigenetic status of multiple gene loci, with changes in both *de novo* methylation and demethylation of DNA and in various histone modifications (Ohgane *et al.,* 2002; Shiota *et al.,* 2002; Eckhardt *et al.,* 2006; Bracken *et al.,* 2006; Squazzo *et al.,* 2006; Oakes *et al.,* 2007; Mikkelsen *et al.,* 2007). There are, indeed, numerous tissue-dependent, differentially DNA-methylated regions (T-DMRs) in the unique sequences including genes and their regulatory elements in the mammalian genome.

In this review, we describe 1) the concepts of the DNA methylation profiles of T-DMRs in normal cells and tissues, and 2) the interplay among epigenetic factors in forming the DNA methylation profiles specific to different cell types.

Tissue-dependent, differentially methylated regions (T-DMRs) in unique sequences in the mammalian genome

DNA methylation in repetitive and unique sequences

A remarkable feature of the mammalian genome is that a large portion is composed of non-genic, repetitive elements including interspersed repeats and satellites: ~49% and 44% in the human (hg18 assembly) and mouse (mm9 assembly) genome respectively (Repeatmasker; http://www.repeatmasker.org/). Over 60% of the CpGs of the mouse genome are methylated, the majority of which are located in repetitive elements (Solage and Cedar, 1978; Gruenbaum *et al*., 1981). DNA methylation of the repetitive elements may function to suppress transposon and enhance genome stability (Yoder *et al*., 1997).

Before the 1980s, several genes had also been found to be methylated (McGhee and Ginder, 1979) but these were considered exceptional loci because the 5-MeC level in the unique sequences was much less than the repetitive sequences (Ehrlich *et al.,* 1982). However, recent studies have revealed that DNA methylation is involved in the regulation of many more genes expressed in a tissue- or cell-type-specific manner, and thus plays a role in differentiation and development (Shiota, 2004; Yagi *et al.,* 2008). The epigenetic dynamics of genes are different from those of bulk DNA. Recently there has been much more attention focused on the epigenetic regulation of genes. Currently there are ~25,000 genes annotated in the human (hg18) and mouse (mm9) genome, which comprise less than 3% of the genome (UCSC genome browser; http://genome.ucsc.edu/). Thus far, various genes with T-DMRs have been found, and the details are discussed below.

DNA methylation profiles of T-DMRs in normal cells and tissues

Genome-wide studies have shown that the mammalian genome contains numerous T-DMRs in its unique sequences. Various techniques have been utilized to obtain DNA methylation profiles of unique sequences, including: restriction land mark genomic scanning (RLGS) (Ohgane *et al.,* 2002), genome-wide bisulfite sequencing (Eckhardt *el al*., 2006), and microarrays in combination with DNA methylation-sensitive restriction enzyme (Khulan *et al.,* 2005; Yagi *et al.,* 2008) or antibodies against 5-MeC (Weber *et al*., 2005). All of these studies have reported a substantial amount of T-DMRs by comparing DNA methylation profiles of various somatic tissues (Ohgane *et al.,* 2002; Shiota *et al.,* 2002; Kremenskoy *et al.,* 2003; Song *et al.,* 2005; Khulan *et al.,* 2006; Eckhardt *et al.,* 2006; Sakamoto *et al*., 2008; Yagi *et al.,* 2008), stem cells (Shiota *et al.,* 2002; Kremenskoy *et al.,* 2003), germ cells (Shiota *et al.,* 2002; Oakes *et al.,* 2007; Weber *et al*., 2007), primary cell types (Weber *et al*., 2005; Eckhardt *et al.,* 2006; Weber *et al*., 2007), and cells of different sex or ages (Weber *et al*., 2005; Eckhardt *et al.,* 2006). For example, RLGS with *Not* I, a methylation-sensitive restriction enzyme, was used to analyze the genome-wide DNA methylation status (~1,500 loci) in mouse embryonic stem (ES) cells, embryonic germ (EG) cells, trophoblast stem (TS) cells, germ cells and several somatic tissues, which identified 247 T-DMRs where the methylation status is dependent on the cell or tissue type (Shiota *et al.,* 2002). Taken together, each cell/tissue type has a specific methylation profile of T-DMRs, and these profiles can be used to distinguish between cell/tissue types (Fig. 1).

Based on the fact that approximately 70% of the *Not* I sites were located within CpG islands in the mouse genome (Fazzari and Greally, 2004), it suggested that CpG islands had T-DMRs. However, this type of T-DMRs represents a relatively small proportion (Eckhardt *et al.,* 2006; Sakamoto *et al.,* 2007). In addition, T-DMRs within CpG islands are generally restricted to subregions. For example, the T-DMR of the *Sphk1* gene, which is conserved in the mouse, rat and human, occupies ~200 base pairs (bp) at the edge of a 3.7-kilo bp CpG island, a fraction of less than 10% (Imamura *et al.,* 2001). This type of T-DMR, which is restricted to a small CpG island region, has also been found in the *EDNRB* and *POMC* genes (Shiota, 2004). On the other hand, all CpGs in the CpG island are methylated in the *Tact1/Actl7b* and *Ant4/Slc25a31* genes in the somatic tissues, whereas they are unmethylated in germ cells (Hisano *et al.,* 2003; Suzuki *et al.,* 2007). Therefore, there is a novel type of CpG island T-DMR, in which all of the CpGs are fully methylatable in normal cells. Recent genome-wide analyses have confirmed that regions with both lower CpG content as well as those having higher CpG frequencies, including CpG islands, were in fact methylated (Eckhardt *et al.,* 2006; Weber *et al.,* 2007; Sakamoto *et al.,* 2007; Fouse *et al.,* 2008). Collectively, there are T-DMRs associated with genes both in CpG-poor and CpG-rich sequences. In the near future, the number of T-DMRs will expand with additional studies; consequently, the DNA methylation profile in the entire genome will become more complex.

The DNA methylation profile is specific to cell and tissue type,

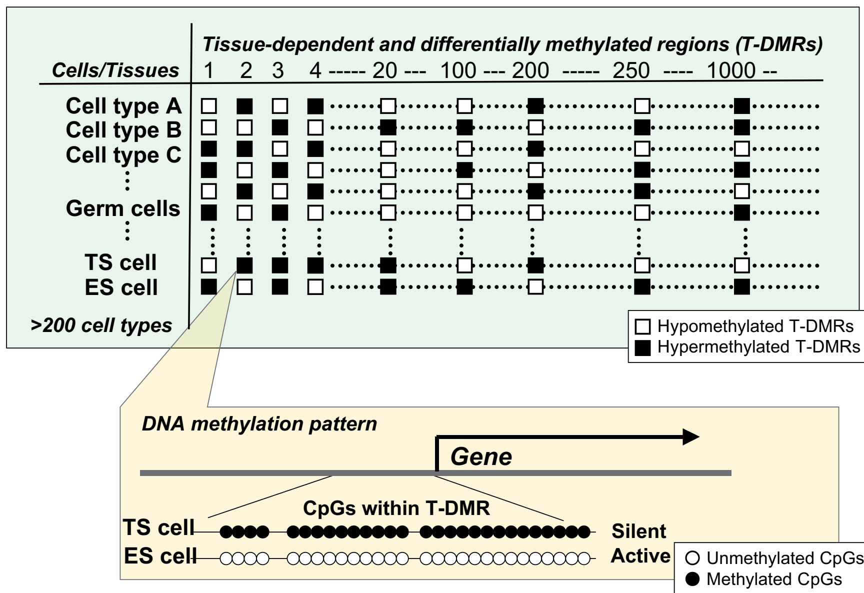

Fig. 1. Cell-type-specific DNA methylation profiles consisting of differentially methylated T-DMRs. *Each cell type (ES cells and TS cells) has a unique pattern of DNA methylation at CpGs within T-DMRs (lower panel). These differential methylation status in T-DMRs constitute cell-type-specific DNA methylation profiles genome-wide (upper panel). The DNA methylation profile of a cell type can be used as a novel tool to define and characterize the cell type.*

much like a fingerprint is distinct, and can be used as an identification (ID) tag for cells (Fig. 1). The DNA methylation profile also provides a novel method to evaluate the similarities of cells because it reflects the similarity in pattern of differentiation lineages (Sakamoto *et al.,* 2007). Since DNA methylation profile is a unique ID of cells, a change in the DNA methylation profile will cause an alteration in cell properties.

Epigenetic system of DNA methylation and histone modification

DNA methylation, histone modification and chromatin structure

DNA methylation status is closely associated with chromatin structure (Fig. 2). It is known that the micrococcal nuclease-resistant closed chromatin fraction predominantly contains 5-MeC (Solage and Cedar, 1978). Histone modification also influences chromatin structure. Acetylation of lysine residues is thought to directly neutralize the positive charge of histones and contributes to a relaxation of the interaction between DNA and histones. Although this is not always the case, *in vitro* study showed that acetylation at K16 of histone H4 was sufficient to inhibit the formation of the 30 nanometer-like chromatin fibers (Shogren-Knaak *et al.,* 2006). Another route mediates proteins recognizing histone modifications including chromodomain and bromodomain proteins, which bind to methylated and acetylated lysines, respectively, and affect chromatin structure as a member of the transcriptional repressor or chromatin remodeling complexes (Kouzaridez, 2007). Acetylation of histones H3/H4 (H3Ac, H4Ac) and di- and trimethylation of lysine 4 of H3 (H3K4me2 and me3) are generally found in transcriptionally active, nucleosome-depleted, and nuclease-sensitive chromatin (Birney *et al.,* 2007). In contrast, methylation of K9 and K27 of histone H3 (H3K9me and K27me) has usually been found in transcriptionally silent chromatin regions (Lachner *et al.,* 2003). The profile of histone modifications in the non-repetitive regions of the genome is also unique to the tissue- or cell-type (Hattori *et al.,* 2004b; Bracken *et al.,* 2006; Squazzo *et al.,* 2006; Tomikawa *et al.,* 2006; Mikkelsen *et al.,* 2007).

Epigenetic control of stemness-related genes (Oct-4 and Nanog)

In mammalian embryogenesis, the first differentiation event that determines the lineage of the trophectoderm and inner cell mass (ICM) occurs at the blastocyst stage. ES cells established from ICM have a pluripotent ability to contribute to all embryonic lineages (Martin, 1981). TS cells established from trophectoderm have the ability to differentiate into the trophoblast lineage *in vitro* (Tanaka *et al.,* 1998). The *Oct-4* gene is a POU family transcription factor and has a CG-rich and TATA-less promoter. *Oct-4* is expressed in ES cells but not in TS cells (Tanaka *et al.,* 1998), and reduction in *Oct-4* gene expression induces the transdifferentiation of ES cells into TS-like cells under certain culture conditions (Niwa *et al.,* 2000). There is a T-DMR in the promoter and enhancer region of the *Oct-4* gene and the T-DMR is hypomethylated in ES cells, but hypermethylated in TS cells (Hattori *et al.,* 2004b).

DNA hypomethylation of the T-DMR of the *Oct-4* gene is associated with hyperacetylated histones in ES cells, implying that DNA methylation status is correlated with chromatin structure (Hattori *et al.,* 2004b). Nanog is another transcription factor that is related to the stemness of preimplantation embryo and ES cells. The *Nanog* gene also has a T-DMR with relatively rich CpG sequences (Hattori *et al.,* 2007). Similar to *Oct-4*, the *Nanog* T-DMR is hypermethylated in TS cells and hypomethylated in ES cells. The *Nanog* T-DMR is also hyperacetylated in ES cells and hypoacetylated in TS cells. In the *Nanog* T-DMR of TS cells, lysines 9 and 27 (K9 and K27) of histone H3 are hypermethylated. In contrast, the methylation of both K9 and K27 is low in the *Oct-4* T-DMR. Thus, these genes are regulated by an intimate relationship between DNA methylation and histone modifications in distinct combinations.

Epigenetic factors involved in epigenome formation

The mammalian genome displays a complex profile of DNA methylation and histone modifications, referred to as the epigenome. The profiles clearly show that differentiation involves massive alterations of the genome-wide epigenetic status of multiple gene loci, with changes in both DNA methylation and various histone modifications (Ohgane *et al.,* 2002; Shiota *et al.,* 2002; Eckhardt *et al.,* 2006; Bracken *et al.,* 2006; Squazzo *et al.,* 2006; Oakes *et al.,* 2007; Mikkelsen *et al.,* 2007). The question is how these profiles are established in the proper cell-types at the appropriate times and how this information is inherited in subse-

quent cellular generations. Taking recent reports into account, the interplay between DNA methylation and histone modifications is one of the mechanisms underlying the epigenetic memory (Birke *et al.*, 2002; Fujita *et al.*, 2003; Fuks *et al.*, 2003a; Esteve *et al.*, 2006; Li *et al.*, 2006a; Vire *et al.*, 2006; Ikegami *et al.*, 2007). These epigenetic factors and their interplay are summarized in this section (Fig. 2).

DNA methyltransferases

In mammals, there are three functional DNA methyltransferases (Dnmts), Dnmt1, Dnmt3a and Dnmt3b. Dnmt1 and Dnmt3b knockout mice are embryonically lethal at embryonic day (E) 8.5 and 14.5-18.5, respectively, while those for Dnmt3a die at 4 weeks of age (Li *et al.*, 1992; Okano *et al.*, 1999), indicating the essential role of DNA methylation in mammalian development. Dnmts have been classified into two groups based on their substrate preferences. Dnmt1 preferentially adds methyl group to hemi-methylated DNA (methylated at only one of the double strands) compared with the unmethylated form *in vitro* (Gruenbaum *et al.*, 1982). This implies that Dnmt1 methylates the daughter strand during replication to maintain the parental DNA methylation pattern. In fact, Dnmt1 localizes at replication foci during the S phase (Leonhardt *et al.*, 1992) and directly interacts with methyl-CpG-binding protein 2 (MeCP2), which recognizes hemi-methylated DNA (Kimura and Shiota, 2003). Hence, Dnmt1 has been called "maintenance methyltransferase" for contributing to the inheritance of DNA methylation marks over generations. The other class consists of Dnmt3a and Dnmt3b, which prefer unmethylated DNA as a substrate *in vitro* (Okano *et al.*, 1998). In addition, the double knockout of Dnmt3a and Dnmt3b prevents *de novo* DNA methylation induced by viral infection. Hence, Dnmt3a and Dnmt3b have been termed "*de novo* methyltransferases" (Okano *et al.*, 1999).

However, such classical groupings like maintenance and *de novo* methylation enzymes may not be adequate to understand the intracellular mechanisms, because there is direct interaction among DNMT1, 3A and 3B *in vivo*, as demonstrated in human cells (Kim *et al.*, 2002). Knockout of both Dnmt3a and Dnmt3b shows progressive loss of DNA methylation at repetitive sequences with the increasing number of passages, indicating that Dnmt3a and Dnmt3b also function as "maintenance methyltransferases" (Chen *et al.*, 2003). A number of normally methylated unique sequences cause drastic loss of DNA methylation in Dnmt3a and Dnmt3b double knockout ES cells, while only a partial reduction was achieved in Dnmt1 knockout cells (Hattori *et al.*, 2004a). In contrast, DNA methylation levels at repetitive sequences are

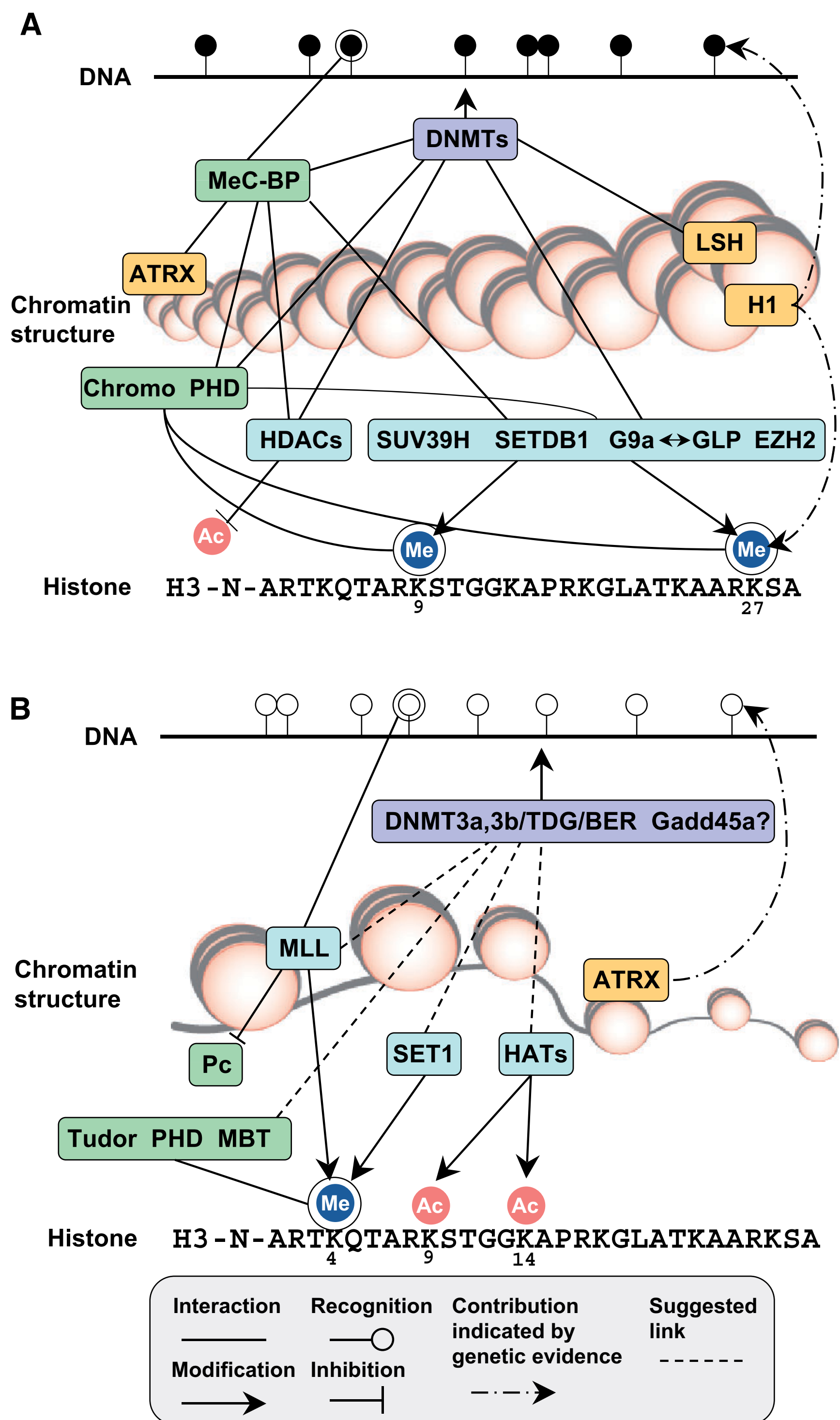

Fig. 2. Molecular link between DNA methylation, chromatin structure and histone modifications in inactive (A) and active (B) genomic regions. *BER, base excision repair proteins (e.g. AP endonuclease, DNA polymerase I); Chromo, chromodomain proteins (e.g. HP1, Pc); DNMTs, DNA methyltransferases (e.g. Dnmt1, Dnmt3s); HATs, histone acetylases (e.g. CBP/P300); HDACs, histone deacetylases (e.g. HDAC1, HDAC2); MBT, MBT domain proteins; MeC-BPs, methylCpG binding proteins (e.g. MBDs, MeCP2, UHRF1); PHD, PHD finger proteins (e.g. BPTF, SMCX); TDG, thymine DNA glycosylase; Tudor, Tudor domain proteins.*

severely affected by Dnmt1 deficiency (Li *et al.,* 1992). Therefore, Dnmts have preferences for certain target sequence characteristics, rather than a sequential process of establishment and maintenance of DNA methylation by Dnmt3a/3b followed by Dnmt1.

A recent study showed that Dnmt3a/3b are able to deaminate 5-MeC in double-stranded DNA, which generates a T:G mismatch (Metivier *et al.,* 2008). Coordinate recruitment of Dnmt3a/3b, thymine DNA glycosylase (TDG) and base excision repair (BER) proteins occurred during DNA demethylation of an estrogen receptor alpha (ERα) target gene, pS2 promoter, which exhibited periodic DNA methylation/demethylation during transcriptional cycling induced by estrogen (Metivier *et al.,* 2008; Kangaspeska *et al.,* 2008). These results suggest that Dnmt3a/3b, in cooperation with TDG and BER proteins, plays an active role in DNA demethylation. DNA demethylation has also been proposed to involve nucleotide excision repair mediated by Gadd45a (Barreto *et al.,* 2007), although this mechanism is still controversial (Jin *et al.,* 2008).

Histone H3 lysine 9 methyltransferases

Five histone H3 lysine 9 (H3K9) methyltransferases have been identified in mammals. These histone methyltransferases (HMTs) have a ~130 amino acid length SET (Su(var), E(z), Trithorax) domain responsible for lysine methylation activity. A K9 residue is modified as mono- (K9me1), di- (K9me2) or trimethylation (K9me3).

Suv39h1 and Suv39h2 are H3K9 HMTs, which predominantly localize to the DAPI-dense heterochromatin region (Aagaard *et al.,* 1999). Suv39h1 or Suv39h2 single knockout mice display normal viability and fertility, but double mutants are born at sub-Mendelian ratios with growth retardation and male sterility (Peters *et al.,* 2001). The Suv39h1/h2 double null exhibits loss of H3K9me3 at pericentromeric heterochromatin and chromosome missegregation in the embryonic fibroblasts, indicating that their catalytic activities are crucial for heterochromatin organization (Peters *et al.,* 2001). It is also known that Suv39h1, together with retinoblastoma (Rb) protein and heterochromatin protein 1 (HP1), represses genes located in euchromatin (Nielsen *et al.,* 2002).

G9a (officially Ehmt2) is another H3K9 HMT, which distributes in the nucleus except for pericentromeric heterochromatin (Tachibana *et al.,* 2001; Tachibana *et al.,* 2002). Knockout of G9a protein in ES cells exhibits drastic loss of H3K9me1 and H3K9me2 in euchromatin, indicating that G9a is a mono- and dimethyltransferase (Tachibana *et al.,* 2002; Tachibana *et al.,* 2005). An *in vitro* study has indicated that G9a can also transfer three methyl groups to a lysine residue, although this reaction is slower than dimethylation (Patnaik *et al.,* 2004).

G9a associates with transcriptional repressors (Shi *et al.,* 2003) and contributes to transcriptional silencing (Tachibana *et al.,* 2002). Another report has shown that G9a also participates in transcriptional activation independent of HMT activity (Lee *et al.,* 2006). G9a is expressed ubiquitously in somatic tissues with a high level in the testis, where G9a has a crucial role in germ cell development (Tachibana *et al.,* 2005; Tachibana *et al.,* 2007). G9a is also essential for early embryogenesis, because G9a knockout mice are embryonically lethal (E9.5; Tachibana *et al.,* 2002).

GLP, (G9a-like protein; officially Ehmt1), is a H3K9 HMT structurally similar to G9a, forms a functional heterodimer with G9a and distributes in euchromatin. GLP deficiency is sufficient to cause drastic loss of H3K9me2 and reactivates a known G9a target gene (Tachibana *et al.,* 2005). GLP knockout mice die around E9.5 with almost an identical phenotype to that of G9a (Tachibana *et al.,* 2005). However, expression pattern of GLP is slightly different from that of G9a, displaying higher and lower expression in ES cells and testis than G9a, respectively (Tachibana *et al.,* 2005). Although only G9a but not GLP is present in primordial germ cells (PGCs) at around E7.5, these cells retain H3K9me2 in the nucleus (Seki *et al.,* 2007). Thus, these proteins may function independently of each other in certain circumstances.

SETDB1/ESET is a euchromatin-distributed H3K9 HMT (Schultz *et al.,* 2002). SETDB1 catalyzes H3K9me2 and H3K9me3, and mAM, a murine ATFa-associated factor AM (officially Atf7ip), facilitates SETDB1 to convert di- to trimethylation *in vitro* and in an artificial system *in vivo* (Wang *et al.,* 2003). SETDB1 participates in gene silencing with transcriptional repressors/corepressors including KAP-1, mAM, mSin3A/B (Schultz *et al.,* 2002; Wang *et al.,* 2003; Yang *et al.,* 2003). SETDB1 deficiency exhibits a more severe phenotype in the mouse than those of Suv39h, G9a and GLP HMTs, as it results in peri-implantation lethality between E3.5 and 5.5, and knockout ES cells cannot be derived (Dodge *et al.,* 2004).

Histone H3 lysine 27 methyltransferases

Ezh2 is a SET domain protein catalyzing methylation of histone H3 at lysine 27 (H3K27) (Cao *et al.,* 2002). Ezh2, together with Eed, Suz12 and Rbbp4/7 forms Polycomb repressive complex 2 (PRC2), which is liked to X inactivation and cooperates with PRC1 to act as a transcriptional repressor of multiple developmental genes including homeobox genes (Schuettengruber *et al.,* 2007). Either Eed or Suz12 deficiency causes a sharp reduction of the Ezh2 and H3K27 methylation levels, indicating that Suz12 and Eed have a crucial role in the methyltransferase activity of Ezh2 (Pasini *et al.,* 2004; Montgomery *et al.,* 2005). Interestingly, Eed knockout exhibits loss of H3K27me1, K27me2 and K27me3, whereas that of Suz12 results in reduction of only K27me2 and K27me3, suggesting that Eed may contribute to the substrate-specificity of Ezh2 (Pasini *et al.,* 2004; Montgomery *et al.,* 2005).

Ezh2 knockout mice exhibit peri-implantation lethality (~E7.5), and mutant ES cells cannot be derived from blastocysts, indicating that Ezh2 plays a crucial role in early development such as gastrulation (O'Carroll *et al.,* 2001). Likewise, both Suz12 knockout and Eed knockout mice display embryonic lethality around E7.5-8.5 (Faust *et al.,* 1995; Pasini *et al.,* 2004). Thus far, genome-wide studies have revealed that there are many PRC2 target genes which overlap with H3K27me3 in the mammalian genome in stem and somatic cells (Boyer *et al.,* 2006; Bracken *et al.,* 2006; Lee *et al.,* 2006; Squazzo *et al.,* 2006).

G9a and GLP also exhibit methyltransferase activity for H3K27, although this is weaker than that for H3K9 *in vitro* (Tachibana *et al.,* 2001; Tachibana *et al.,* 2005). G9a deficiency does not alter the global level of H3-K27 methylation in ES cells (Peters *et al.,* 2003). Rougeulle *et al.* (2004) did not detect reduction of H3K27 methylation at the *Xist* gene on the male X-

chromosome in G9a knockout ES cells. However, G9a target regions, in which G9a deficiency causes DNA hypomethylation, display a decreased H3K9me2 as well as H3K27me2 levels in G9a knockout ES cells (Ikegami *et al.,* 2007). This finding suggests that G9a also catalyzes H3K27me2 *in vivo*.

DNA methylation directs histone H3K9 methylation

The *Arabidopsis* mutant null for MET1, a DNMT1-like CpG methyltransferase, displays drastic loss of H3K9 methylation in heterochromatic regions including transposons (Tariq *et al.,* 2003). In the human cancer cell line, deficiency of DNMT1 exhibits global reduction of H3K9me2 and H3K9me3, and transfection of murine Dnmt1 rescues this phenotype (Espada *et al.,* 2004). Interestingly, this DNMT1 knockout cell line displays increased sensitivity to micrococcal nuclease digestion, indicating the role of DNMT1 in chromatin organization. Similarly, knockdown of DNMT1 by siRNA causes reduction in H3K9me1, K9me2 and K9me3 levels at intergenic spacer elements of ribosomal DNA (rDNA) (Esteve *et al.,* 2006). However, Dnmt1/Dnmt3a/Dnmt3b triple knockout ES cells, which exhibit complete loss of DNA methylation at several repetitive sequences and imprinting genes, do not exhibit reduction of the global levels of H3K9me2 and H3K9me3 nor alter localization pattern of H3K9me3 and HP1β (Tsumura *et al.,* 2006).

Histone H3K9/K27 methylation directs DNA methylation

The *Neurospora crassa* mutant of the gene encoding Dim-5, a H3K9 HMT, exhibits a decrease in DNA methylation levels in specific regions including rDNA genes and transposons (Tamaru and Selker, 2001). In *Arabidopsis*, a mutation of the H3K9 methyltransferase *KRYPTONITE* gene results in a reduction of asymmetric CpNpG methylation levels (where N is A, C, G, or T) (Jackson *et al.,* 2002). In mouse ES cells, double null of Suv39h1 and Suv39h2 causes a reduction of DNA methylation levels in major satellite repeats of the pericentromeric region (Lehnertz *et al.,* 2003). ES cells lacking G9a exhibit a reduction of DNA methylation together with H3K9 methylation at the known imprinting gene *Snrpn* (Xin *et al.,* 2003). RNA interference of EZH2 reduces H3K27 methylation levels and DNA methylation levels at known EZH2 target genes (Vire *et al.,* 2006).

SETDB1 may also affect DNA methylation because SETDB1 interacts with MBD1 and DNMT3A (Sarraf *et al.,* 2004; Li *et al.,* 2006a) (Discussed below). However, knockout of SETDB1 in blastocysts did not change the DNA methylation levels at the interspersed repetitive elements (Dodge *et al.,* 2004).

Genome-wide DNA methylation analysis of ES cells revealed that G9a deficiency causes reduction of DNA methylation levels at 1.6% (32 loci) of the total of ~2,000 genic loci analyzed (Ikegami *et al.,* 2007). At these regions, G9a knockout ES cells exhibit reduced levels of H3K9me2 and/or H3K27me2, and the G9a-transgene rescues the DNA methylation status in G9a knockout cells. Importantly, however, DNA methylation levels remained intact at a majority of the genic loci in the G9a knockout ES cells. Therefore, G9a participates in DNA methylation in a locus-specific manner (Ikegami *et al.,* 2007).

Collectively, these reports show that H3K9 and K27 methylation can direct DNA methylation of gene regions, providing clues for how local DNA methylation or demethylation occurs when Dnmts, the global regulators, establish the genome-wide DNA methylation profile.

Interaction between H3K9/27 HMTs and Dnmts

Co-expression study revealed that human SUV39H1 binds to murine Dnmt1 and Dnmt3a (Fuks *et al.,* 2003a). In a breast cancer cell line, sequential ChIP analysis with antibodies against DNMT3A and SETDB1 revealed that SETDB1 binds to DNMT3A on chromatin (Li *et al.,* 2006a). Direct interaction between H3K27 methyltransferase EZH2 and DNMTs (DNMT1, 3A and 3B) was also reported (Vire *et al.,* 2006), supporting the notion that H3K27 methylation links to DNA methylation. Furthermore, DNMTs associate with EZH2-target genes in the presence of EZH2 (Vire *et al.,* 2006).

G9a also interacts with DNMT1 and co-localizes specifically in the S-phase of synchronized human cells (Esteve *et al.,* 2006). In the report, both G9a and DNMT1 localized at replication sites, as judged by strong BrdU incorporation. Therefore, G9a and DNMT1 may cooperatively maintain the H3K9 methylation and the DNA methylation pattern at replication foci.

The molecular interaction between the proteins directly involved in DNA methylation and H3K9/K27 methylation suggests that both modifications could be the basis for recruiting the other.

Proteins recognizing DNA methylation

The human genome encodes 12 proteins containing the methyl-CpG-binding domain (MBD), which is the protein motif responsible for binding to methylated CpG dinucleotide (http://www.ebi.ac.uk/interpro). Among them, MeCP2, MBD1, MBD2, and MBD4 have been shown to specifically recognize methylCpG (Klose and Bird, 2006). In contrast to animals deficient for Dnmts and HMTs, mutant of each of these 4 MBD proteins survives to adulthood (Fatemi and Wade, 2006). However, mutation of the X-linked *MeCP2* gene causes the neurological disease Rett syndrome almost exclusively in females, which results in developmental arrest between 6 and 18 months of age in human (Amir *et al.,* 1999).

Kaiso, (officially Zbtb33), Zbtb4 and Zbtb38 use an ancestral three-zinc-finger motif to bind to methylCpG, but are differentially expressed in mouse tissues, suggesting nonoverlapping functions or targets (Prokhortchouk *et al.,* 2001; Filion *et al.,* 2006). UHRF1 (ubiqutin-like, containing PHD and RING finger domains 1), a binding partner of Dnmt1, preferentially binds to hemi-methylated DNA via its SRA (SET and RING associated) domain (Bostick *et al.,* 2007). Knockout of *Uhrf1* gene leads to mislocalization of Dnmt1 in S phase and reduction of DNA methylation levels, suggesting a role of UHRF1 in maintaining DNA methylation patterns (Bostick *et al.,* 2007; Sharif *et al.,* 2007).

Proteins recognizing histone methylation

Chromodomain recognizes methylation of lysines 9 and 27, which reside within a highly related sequence motif Ala-Arg-Lys-Ser (ARKS). HP1 proteins (HP1α,β and γ) bind to methylated H3K9 via their chromodomains (Lachner *et al.,* 2001). HP1 also directly binds to SUV39H1 (Aagaard *et al.,* 1999) and G9a (Sampath *et al.,* 2007). Another chromodomain protein, Polycomb (Pc), a member of PRC1, binds to methylated H3K27 (Fischle *et*

al., 2003). Biochemical assay and crystallography demonstrated that *Drosophila* HP1 and Pc specifically interact with H3K9me3 and H3K27me3, respectively (Fischle *et al.,* 2003). However, in mice, none of the five murine Polycomb homologs (Cbx2, Cbx4, Cbx6, Cbx7, and Cbx8) has exclusive preference for K27me3 (Bernstein *et al.,* 2006). Instead, Cbx2 and Cbx7 display strong affinity for both K9me3 and K27me3, and Cbx4 inversely prefers K9me3 similar to HP1 (Bernstein *et al.,* 2006). HP1 and Cbx4 might have a role in the discrimination of H3K9 from H3K27 methylation in mammals.

Tudor domains that are structurally related to chromodomain, bind to methyllysines. JMJD2A recognizes H3K4/H3K20me3 through the domain, and also demethylates H3K9/H3K36me3 via jumonji C (jmjC) domain (Huang *et al.,* 2006; Klose *et al.,* 2006). Proteins having a MBT (malignant brain tumor) domain, which is also chromodomain-related, have a broader spectrum of target lysines including H3K4, H3K9, H3K27, H3K36, H4K20 and K26 of histone H1 isotype b (Trojer and Reinberg, 2008). Another type, PHD (plant homeodomain) finger domain is structurally nonrelated to chromodomain. Various PHD finger proteins involving transcriptional activation, repression, and recombination have been reported to recognize H3K4me3 (Kouzarides *et al.,* 2007). More recently, SMCX (officially JARID1C), a jmjC domain-containing H3K4me3 demethylase, is reported to bind H3K9me3 via PHD domain (Iwase *et al.,* 2007).

Proteins connecting DNA methylation and histone methylation

The MBD proteins have a role in connecting DNA methylation to histone methylation. MBD1 directly interacts with Suv39h1 *in vitro* and colocalizes with Suv39h1 *in vivo* in a MBD-dependent manner (Fujita *et al.,* 2003). MBD1 also interacts with HP1 (Fujita *et al.,* 2003). Similarly, MeCP2 has been shown to associate with H3K9 methyltransferase activity, although the exact partner(s) have not been identified (Fuks *et al.,* 2003b). Therefore, MBD proteins play a role in recruiting H3K9 methylation to the site of DNA methylation, and/or HMT recruits MBD proteins to facilitate their binding to methylated CpG. Recently, the *in vivo* interaction of MBD1 with SETDB1 has been demonstrated (Sarraf *et al.,* 2004). During replication, chromatin assembly factor-1 (CAF-1, officially CHAF1), a histone chaperone complex interacting with PCNA, joins to form the S-phase-specific MBD1/SETDB1/CAF-1 complex (Sarraf *et al.,* 2004). MBD1 may participate in maintaining the H3K9 methylation pattern along with that of DNA methylation at replication foci. Finally, since SETDB1 itself has a MBD motif, the internal MBD may direct H3K9 methylation at CpG methylated genomic regions.

HP1 proteins are linked to the DNA methylation system. HP1 proteins interact with DNMT1, DNMT3A and DNMT3B *in vitro* or on co-expression analysis (Fuks *et al.,* 2003a; Smallwood *et al.,* 2007). *In vivo*, HP1α interacts with Dnmt3b but does not interact or very weakly with Dnmt1 and Dnmt3a (Lehnertz *et al.,* 2003). Therefore, HP1 may recruit Dnmts where H3K9 is methylated. HP1 proteins seem to localize differentially in the nucleus during the cell cycle. For example, HP1β localizes most preferentially among the three HP1s to the centromere during the interphase, whereas only HP1α localizes to the centromere during the metaphase in human cells (Hayakawa *et al.,* 2003). Thus, HP1 may affect localization of Dnmt during the cell cycle. HP1 proteins also directly interact with MeCP2 in an overexpression experiment (Agarwal *et al.,* 2007). These results suggest that H3K9 methylation recruits DNA methylation via HP1 proteins, and that HP1 localization is influenced by DNA methylation pattern via MBD proteins.

DNA methylation and other histone modifications

In mammals, there are at least ten known or predicted H3K4 methyltransferases, which are generally categorized into the MLL (mixed lineage leukemia) family, Set1 family and others (Ruthenburg *et al.,* 2007). MLL, a H3K4 HMT, is a member of the trithorax group, which in general functions antagonistically against polycomb group proteins. At a trithorax/polycomb target gene, *Hox c8*, MLL deficiency causes reductions of the expression and H3K4 methylation levels together with an increased DNA methylation level in a mouse embryonic fibroblast cells (Milne *et al.,* 2002). Similarly, mice lacking the SET domain of MLL display decreased H3K4me1 and increased DNA methylation levels in the *Hox d4* gene (Terranova *et al.,* 2006). Interestingly, this mutant does not exhibit a global change of H3K4me1 and DNA methylation, indicating that H3K4 methylation affects DNA methylation in a locus-specific manner. This notion is supported by the recent genome-wide study demonstrating that there are substantial amount of genes marked by H3K4me3 with either DNA hyper- or hypomethylation at promoters (Fouse *et al.,* 2008). It is noteworthy that MLL shares homology with Dnmt1 in the methyltransferase domain, which selectively binds to unmethylated CpG sequences *in vitro* (Birke *et al.,* 2002). However, forced expression of exogenous MLL in MLL knockout cells does not reduce DNA methylation levels (Milne *et al.,* 2002). Therefore H3K4 methylation may protect certain loci from DNA methylation.

Histone deacetylase 1 (HDAC1) associates with MeCP2 in a corepressor complex, providing a model in which the loci with hypermethylated CpGs undergo histone deacetylation by this complex (Nan *et al.,* 1998). In addition, HDAC1 and HDAC2 directly interact with DNMT1 (Fuks *et al.,* 2003b; Robertson *et al.,* 2000; Rountree *et al.,* 2000). Treatment of *Neurospora crassa* with Trichostatin A (TSA), an inhibitor of histone deacetylase, causes reduction of DNA methylation in several genes, whereas it does not affect the global DNA methylation in repetitive sequences (Selker, 1998). In the human colorectal carcinoma cell line, TSA treatment does not affect the DNA methylation status of the CpG islands of two silent genes that were heavily methylated (Cameron *et al.,* 1999). TSA treatment reduces mRNA and protein levels of DNMT3B in human endometrial cells and those of DNMT1 in Jurkat T cells, respectively (Xiong *et al.,* 2005; Januchowski *et al.,* 2007). Therefore, there seems to be in each a dependency between the histone acetylation and DNA methylation.

Molecular link among DNA methylation, chromatin organization and non-coding RNAs

Chromatin remodeling factors, linker histone and DNA methylation

Other than histone modifications, several proteins associated with chromatin have been reported to be linked with DNA methylation (Fig. 2). One is Lsh (lymphoid specific helicase, officially Hells), a member of the SNF2 ATPase/helicase family, which is

involved in chromatin remodeling. Tissues from Lsh knockout mice exhibit DNA hypomethylation at satellite and interspersed repetitive sequences as well as unique sequences including the tissue-specific genes, *beta-Globin* and *Pgk-2* (Dennis *et al.,* 2001). Lsh interacts with Dnmt3a and Dnmt3b, but not with Dnmt1 (Zhu *et al.,* 2006). DNA demethylation at repetitive sequences appears more severe in Lsh knockout cells than that in Dnmt3a/Dnmt3b double knockout cells, suggesting that Dnmt1 activity is also influenced by Lsh (Dennis *et al.,* 2001). Dnmts may require the chromatin remodeling function of Lsh for their catalytic activities.

ATRX (X-linked alpha thalassemia/mental retardation) is another member of the SNF2 family of ATPase/helicase proteins. Patients having mutation in the ATRX gene display DNA hypomethylation at rDNA and hypermethylation at a satellite repeat (Gibbons *et al.,* 2000). The ATRX protein interacts with MeCP2 in co-transfected cells, and both proteins colocalize at the DAPI-dense heterochromatin domain in the adult brain of the mouse (Nan *et al.,* 2007). In MeCP2-null brain, ATRX delocalizes from heterochromatin (Nan *et al.,* 2007). These reports suggest that DNA methylation and ATRX chromatin remodeling affect each other.

Several reports have demonstrated that linker histone H1 preferentially binds to methylated DNA (Levine *et al.,* 1993; McArthur and Thomas, 1996), whereas others have reported that DNA methylation does not affect H1 binding (Campoy *et al.,* 1995; Nightingale and Wolffe, 1995). Recent fluorescence recovery after photobleaching (FRAP) analysis showed that Dnmt3a/Dnmt3b double knockout causes a delay of the recovery kinetics of linker histones, H1 and H5, in ES cells (Gilbert *et al.,* 2007). DNA methylation seems to facilitate linker histone assembly *in vivo*.

Conversely, histone H1 influences DNA methylation status as well. Knockout of three H1 isotypes (H1c, H1d, and H1e) exhibiting global changing of chromatin structure induces DNA demethylation at specific genes but not satellite or interspersed repetitive sequences (Fan *et al.,* 2005). This mutant also displays global reduction of H3K12 acetylation and H3K27me3.

Collectively, proteins associated with chromatin affect establishment of DNA methylation pattern directly or indirectly via histone modifications, and inversely, DNA methylation influences the distribution and/or function of these proteins.

Higher-order chromatin and DNA methylation

DNA methylation has a role in the organization of higher-order chromatin structure. Dnmt3b-deficient murine embryonic fibroblast cells exhibit reduced DNA methylation levels at endogenous repetitive sequences and a higher rate of polyploidy and aneuploidy in culture (Dodge *et al.,* 2005). Lymphoblastoid cell lines established from patients with immunodeficiency syndrome, centromeric region instability, and facial anomalies (ICF syndrome), which is caused by mutations in the *DNMT3B* gene, also display chromosomal abnormalities including metaphase chromosome decondensation, anaphase and/or interphase chromatin bridging, and abnormal chromatin looping (Gisselsson *et al.,* 2005). Dnmt3a/Dnmt3b double null ES cells exhibit increased clustering of pericentromeric heterochromatin into a few large chromocenters (Gilbert *et al.,* 2007). Therefore, DNA methylation contributes to chromosome dynamics. However, triple knockout of Dnmt1, Dnmt3a and Dnmt3b does not affect the chromosome number in murine ES cells (Tsumura *et al.,* 2006).

Non-coding RNA in the directing of DNA methylation and histone modification

Genetic analyses in plants have revealed that non-coding RNA (ncRNA) is involved in DNA methylation, and this system is known as RNA-dependent DNA methylation (Zaratiegui *et al.,* 2007). In human cells, transfection of 21-nucleotide (nt) RNA homologous to the promoter sequence of *EF1A* gene increased the DNA methylation level in the promoter region (Morris *et al.,* 2004). In the *RASSF1A* gene, short hairpin RNAs (shRNA) containing 21-nt sequences complementary to promoter CpG island induced a very low level of or no detectable DNA methylation in the target region depending on the analysis methods (Castanotto *et al.,* 2005). More recently, knockout of the Piwi proteins, MIWI2 and MILI, which bind to 26-31-nt piRNAs (Piwi-interacting small RNAs), has been reported to decrease DNA methylation levels of transposons in murine male germ cells (Aravin *et al.,* 2007; Kuramochi-Miyagawa *et al.,* 2008). These findings suggest an involvement of piRNA in the DNA methylation system.

In contrast, there are reports that ncRNA did not induce DNA methylation. The 19-nt sequence overlapping the transcription start site of the human *PGR* gene reduced the mRNA level but did not induce DNA methylation around the target sites in a human cancer cell line (Janowski *et al.,* 2005). Similarly, 21nt double-stranded RNAs (dsRNAs), which are homologous to the CpG island of the *CDH1* promoter, induced H3K9me2 dimethylation and transcriptional silencing but not DNA methylation (Ting *et al.,* 2005). Furthermore, the RNA-mediated transcriptional repression was accomplished in cells genetically lacking DNMT1 and DNMT3B (Ting *et al.,* 2005). Collectively, RNA-dependent induction of DNA methylation still remains to be addressed in mammals.

As noted above, shRNA-transfected cells exhibit increased H3K9me2 levels at the *CHD1* promoter (Ting *et al.,* 2005). Similarly, 21 nt RNA homologous to the integrated *CCR5* gene promoter induces H3K9me2 at the promoter (Kim *et al.,* 2006). H3K27me3 and EZH2 levels increase at the *RASSF1A* gene promoter in HeLa cells stably expressing shRNA targeting the promoter (Kim *et al.,* 2006). Interaction between long ncRNA and G9a or EZH2 has been recently shown (Nagano *et al.,* 2008; Pandey *et al.,* 2008). These reports suggest that ncRNA plays a role in the H3K9 and K27 methylation system in mammals.

In contrast to the findings above, ncRNAs have also been suggested to trigger demethylation of DNA and H3K9 in mammalian systems. Human cancer cell lines transfected with 21-nt dsRNA targeting the *E-cadherin* promoter induces transcription and decreases the H3K9me2 and H3K9me3 levels in the target region with the CpG island (Li *et al.,* 2006b). Imamura *et al.,* (2004) reported that transfection of 0.2-1.0 kbp antisense ncRNAs corresponding to the CpG island of the *Sphk1* gene induces demethylation of CpG sites and methylation of CC(A/T)GG sites at the T-DMR in the CpG island. Importantly, these antisense RNAs are in fact transcribed from the CpG island in rat tissues (Imamura *et al.*, 2004).

Conclusion

The cell- and tissue-specific expression of genes, including

transcription factors, have traditionally been explained through the regulation by the transcription factors active in the cell/tissue type. However, the regulation by transcription factors is generally temporally constrained and rapidly changeable in response to environmental and extra-cellular stimuli. It is becoming clear that various genes are controlled by epigenetic systems of DNA methylation and histone modifications. DNA methylation profiles are, in a certain sense, the epigenetic memory that is indispensable for cells and tissues to maintain their unique features.

Analysis of DNA methylation profiles provides us a survey of the epigenetic status of the entire genome, because various histone modifications and chromatin remodeling generally reflect the genome-wide DNA methylation status (Fig. 2). We are now in the era of the epigenome, the "Total information of epigenetic marks on the genome". The epigenome connects the genome (DNA sequence) and transcriptome by introducing another layer of genetic control: a stable memory of the genetic activity for numerous genes, which is heritable to the next generation. The epigenome will provide new insight into mammalian development and the differentiation of cells. In addition, epigenetic analysis provides a new paradigm for understanding the normal and abnormal status of cells. Direct/indirect interactions among Dnmts, histone modification enzymes, histone subtypes, chromatin remodeling factors and ncRNAs underlie the establishment of the cell-type specific epigenome.

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