

Epigenetic mechanisms and genome stability

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Abstract Epigenetic marks are well recognized as heritable chemical modifications of DNA and chromatin that induce chromatin structural changes thereby affecting gene activity. A lesser-known phenomenon is the pervasive effects these marks have on genomic integrity. Remarkably, epigenetic marks and the enzymes that establish them are involved in multiple aspects of maintaining genetic content. These aspects include preserving nucleotide sequences such as repetitive elements, preventing DNA damage, functioning in DNA repair mechanisms and chromatin restoration, and defining chromosomal organization through effects on structural elements such as the centromere. This review discusses these functional aspects of epigenetic marks and their effects on human health and disease.

Keywords DNA methylation · Histone modifications · Microsatellite instability (MSI) · DNA repair · Centromere

Epigenetics is broadly defined as the heritable, regulatory elements of a genome exclusive of its primary DNA sequence. Methylation of DNA and post-translational modifications of the core histone proteins are the most well understood epigenetic marks. Together, they organize DNA into chromatin and ensure proper gene regulation. Additionally, epigenetic regulation of eukaryotic genomes has been increasingly shown to impart stability on the DNA sequence and to aid in maintenance of genomic integrity. Epigenetic aberrations causing genetic instability are at the

root of developmental abnormalities such as ICF (immuno-deficiency, centromere instability, and facial anomalies) syndrome, which is characterized by immune defects, heterochromatin instability, and mental retardation, and disease states such as cancer. This review discusses the fundamentals of epigenetics and presents its many roles in maintaining genome stability.

DNA methylation as an epigenetic mark

To regulate genetic information, a cell's immense quantity of DNA must be systematically packaged and organized, a function provided by chromatin structure. Chromatin can be distinguished as heterochromatin, characterized as a highly condensed and transcriptionally inert state, or as euchromatin, a loosely packaged DNA arrangement easily accessible by transcription factors and the transcription machinery. These states are largely determined by covalent modifications of the histone proteins contained within chromatin and by DNA methylation.

DNA methylation is a covalent modification of DNA that, in vertebrates, predominantly occurs at the carbon-5 position of cytosine nucleotides followed by a guanine (CpG). Patterns of DNA methylation are strongly associated with transcriptional repression and are localized to domains of heterochromatin (Miller et al. 1974). On a global level, in differentiated somatic cells, CpG dinucleotides are methylated at about 80%, whereas CpG islands, which contain longer stretches of CpG-containing sequence and are typically associated with gene promoters, often remain unmethylated, presumably to permit gene activation (Bird 1999, 1986; Gardiner-Garden and Frommer 1987; Jaenisch and Bird 2003).

The cell's DNA methylation “signature” provides explicit instructions for directing functions such as maintain-

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ing pluripotency or promoting tissue-specific gene expression and controlling growth and proliferation. Establishment of proper DNA methylation patterns and maintenance of those patterns through mitotic division is critical for cellular function. During mammalian development soon after fertilization, gene expression profiles are “reset” by a wave of widespread DNA demethylation, especially of the paternal genome (Jaenisch and Bird 2003; Mayer et al. 2000; Oswald et al. 2000). Subsequently, genome-wide de novo methylation commences following implantation of the zygote. The DNA methyltransferases (DNMT) DNMT1, DNMT3A, DNMT3B, and DNMT3L actively contribute to and determine these DNA methylation patterns in mammalian cells by differential means. DNMT1 has a strong bias for hemi-methylated DNA and localizes to DNA replication foci; these features are consistent with DNMT1 playing a major role in maintenance of methylation in dividing cells (Bestor 1992; Leonhardt et al. 1992). DNMT3A and DNMT3B, on the other hand, operate equally well on unmethylated or hemi-methylated DNA substrates and thus are classified as possessing de novo methylation activity (Liang et al. 2002; Okano et al. 1999; Yokochi and Robertson 2002). Their activity is enhanced by DNMT3L, a catalytically inactive protein with sequence similarity to DNMT3A and DNMT3B (Chedin et al. 2002; Hata et al. 2002; Jia et al. 2007; Margot et al. 2003; Suetake et al. 2004). Both de novo and maintenance methylation are required for development, since deficiency for any of the DNMTs affects viability (Okano et al. 1999). DNA methylation marks are interpreted by methyl-CpG-binding proteins including MBD1, MBD2, MBD3, and MeCP2, which generally act to silence gene expression and recruit histone deacetylases (HDACs), which are required for chromatin condensation (Bowen et al. 2004; Boyes and Bird 1991; Jaenisch and Bird 2003; Lewis et al. 1992; Meehan et al. 1989; Nan et al. 1997). Thus, DNA methylation and the proteins associated with writing (DNMTs) and reading (MBDs) this mark provide a robust mechanism for regulation of transcriptional activity, but more importantly, the stability of DNA methylation as a covalent modification and the replicable nature of DNA methylation marks facilitate the *inheritance* of epigenetic information during important processes such as X chromosome inactivation, imprinting, silencing of repetitive elements, transposable elements, and pericentromeric repeats, and developmental gene regulation (Gopalakrishnan et al. 2008; Li 2002; Slotkin and Martienssen 2007).

The key question regarding DNA methylation is what factors or stimuli determine how methylation patterns are established. Studies in *Arabidopsis* show that positioning of nucleosomes along DNA is directly related to the patterning of DNA methylation: nucleosome-bound DNA is enriched for methylation compared to nucleosome-

flanking sequences (Chodavarapu et al. 2010). Likewise, the histones comprising the nucleosome core bear post-translational modifications involved in directing DNA methylation patterns, chromatin restructuring, and transcriptional effects (Hu et al. 2009; Ooi et al. 2009, 2007; Zhang et al. 2010).

Histone modifications in chromatin function

The basic repeating unit of chromatin is the nucleosome, containing an octamer of four core histones, H2A, H2B, H3, and H4, around which double-stranded DNA is coiled. Each histone consists mostly of a globular domain except for an N-terminal tail, which protrudes from the nucleosome where it is accessible for binding to DNA, for protein interactions, and for covalent attachment of small molecules. Covalent modifications of amino acid residues in histones direct DNA methylation, chromatin conformation, and gene transcription by promoting or restricting the recruitment of gene regulatory proteins (Jenuwein and Allis 2001). The most well understood histone modifications include methylation, phosphorylation, acetylation, and ubiquitination, and chromatin regulation is conferred by the combination of these modifications on different residues and, in the case of histone methylation, by the number of moieties attached to a specific residue (Kouzarides 2007). Methylation of histone H3 lysine 9 (H3K9), H3K27, H4K20, and H3 arginine 2 (R2) are just a few of the marks determining heterochromatin formation and transcriptional repression (Kirmizis et al. 2007; Kouzarides 2007). Euchromatin is characterized by methylation of H3K4 and acetylation of H3 and H4 histone tails. For histone methylation, the number of attached methyl groups also influences its role as a regulatory mark. Lysines and arginines are mono-, di-, or trimethylated. Di- and trimethylation of H3K4 (H3K4me2 and H3K4me3), for example, is strongly associated with regions of transcriptional permissiveness, and hypomethylation of this residue is correlated with loss of H3K9 acetylation (Bernstein et al. 2002; Santos-Rosa et al. 2002; Schneider et al. 2004). While H3K9me3, H3K27me3, and H4K20me3 are all heterochromatic marks, mono-methylation of these same residues correlates with transcriptional permissiveness. Additionally, some histone marks show specificity for certain gene regions. Transcriptionally active genes are characterized by H3K4me2 within their coding regions, while histone acetylation and H3K4me3 are enriched in promoter regions, and H3K4me1 occurs in enhancer elements (Bernstein et al. 2002; Heintzman et al. 2007; Liang et al. 2004; Schneider et al. 2004). H3R2me2, which is mutually exclusive with H3K4me3, is found within

transcriptionally inactive genes and in the 3' region of transcribed genes (Kirmizis et al. 2007).

A collection of histone-modifying enzymes including lysine methyltransferases, arginine methyltransferases, serine-threonine kinases, and acetyltransferases is involved in writing each mark. These enzymes prove to be highly specific in recognizing a single amino acid substrate, and multiple enzymes provide functional redundancy for writing each mark and/or the ability of particular marks to respond to many different stimuli (Kouzarides 2007). For instance, the SET domain methyltransferases SUV39H1, SUV39H2, G9, and SETDB1, among others, show strict specificity for methylating H3K9. Likewise, mixed lineage leukemia enzymes 1–5 specifically recognize and methylate only H3K4 residues (Kouzarides 2007).

The combinations of these modifications at specific loci are highly dynamic and capable of rapid change depending on a cell's response to cell–cell signaling and environmental cues. For instance, pluripotent cells possess a state of “bivalency” in which H3K27 methylation, a mark of heterochromatin, overlaps with loci containing euchromatic H3K4 methylation (Azuara et al. 2006; Bernstein et al. 2006; Ku et al. 2008). These opposing marks occupy genes encoding many developmentally important transcription factors that were presumably “poised” for either activation or dense repression by stabilization of one of these marks upon differentiation. Thus, some have questioned whether histone modifications should be referred to as epigenetic marks under a strict classical definition of a stable element, faithfully inherited throughout many cell generations (Kouzarides 2007). Recent evidence has shown that at least some histone marks are indeed preserved and replicated during cell division. The repressive mark H3K27me3 is maintained during DNA replication by binding of the polycomb repressive complex 2 to H3K27me3 within promoters of transcriptionally repressed genes (Hansen et al. 2008). Identifying additional mechanisms that allow for the reproduction of histone modifications within the chromatin of newly synthesized DNA will be important for understanding how gene expression programs are controlled. Regardless of their heritability, these modifications provide important information crucial for genomic stability and proper DNA methylation; so, for the purpose of this review, we continue to refer to them as epigenetic marks.

As previously mentioned, histone modifications are important in establishing DNA methylation marks. Repressive histone marks may be established independently of DNA methylation and are capable, in some cases, of inducing de novo DNA methylation. DNMT3L recognizes loci lacking unmethylated H3K4, and an interaction between DNMT3L and the most N-terminal residues of histone H3 is required for de novo DNA methylation (Hu et al. 2009; Ooi et al. 2007). Furthermore, DNMT3A and DNMT3B interact with

SUV39H1 and EZH2, which are H3K9 and H3K27 histone methyltransferases, respectively, as well as residues 1–19 in the N-terminal tail of histone H3 (Lehnertz et al. 2003; Vire et al. 2006; Zhang et al. 2010). Conversely, DNA methylation may also direct establishment of repressive histone marks. In ICF cells deficient for DNMT3B function, loci affected by loss of DNA methylation also demonstrated a loss of repressive H3K27 methylation marks and a coordinated gain of H3K9 acetylation and H3K4 trimethylation (Jin et al. 2008).

Epigenetics and genomic stability

Originally, DNA methylation may have been evolutionarily acquired to defend against genomic disruption by parasitic insertional element translocations in eukaryotic genomes, an idea that has become widely supported (Robertson and Wolffe 2000; Slotkin and Martienssen 2007; Yoder et al. 1997). Subsequently, functionality for DNA methylation in other modes of genomic stability may have been co-opted (Colot and Rossignol 1999). In the following sections, we focus on the evidence that epigenetic mechanisms help to preserve genomic content.

Effects of epigenetic marks on microsatellite repeat stability

DNA methylation and DNA methyltransferases have been implicated in affecting stability of microsatellites within the genome for some time. Microsatellite loci are repetitive sequences typically consisting of one to four nucleotide repeats, and they are particularly susceptible to length change mutations. Microsatellite repeat instability (MSI) presents a notable hazard in light of its involvement in diseases such as Huntington's, myotonic dystrophy, and cancer among others (see below) and is often associated with defects in the mismatch repair (MMR) machinery, a system that specifically recognizes and repairs errors in nucleotide base pairing.

Repetitive regions are subject to expansion or contraction when single-stranded DNA becomes exposed, allowing repetitive sequences to form secondary structures (i.e., DNA hairpins), particularly during replication, transcription, and DNA repair (Pearson et al. 2005). These secondary structures may then become incorrectly resolved through MMR or cause slippage of DNA polymerases along repetitive sequence during replication. For a history of MSI discovery and characterization, see Laghi et al. (2008).

Epigenetic mechanisms appear to protect against MSI both directly and indirectly (Table 1). Disruption of DNA methylation or the DNMTs destabilizes repeats through a

Table 1 Epigenetic mechanisms of genetic instability

Influence on repetitive elements	Epigenetic involvement	Molecular consequence
Direct	Transcriptional repression of repetitive elements by DNA methylation	Prevents DNA damage produced by DNA secondary structures or homologous recombination
	Chromatin remodeling by DNMTs	Chromatin condensation reduces DNA exposure to mutagenic factors or processes
	Involvement of DNMTs in DNA repair	DNMT1 interaction with the MMR machinery facilitates DNA repair
Indirect	Hypermethylation of DNA repair genes (i.e., MMR)	Reduced expression of genes required for genetic stability

direct local chromatin remodeling effect, such as transcriptional de-repression, or possibly another unknown mechanism. Alternately, DNA methylation affects repeat stability secondarily by producing global changes in gene expression that alter expression of proteins responsible for repeat stabilization (i.e., MMR genes). Here, we discuss the evidence for these epigenetic effects on MSI and their potential mechanisms.

Several groups have shown that MSI results from disruption of DNMT1 or of DNA methylation patterns. Most notably, *DNMT1* mutants were isolated from an insertional mutagenesis screen for ES cell clones exhibiting defects in MMR (Guo et al. 2004). To measure MSI in these mutants, a “slippage” assay was employed in which an insertional construct containing repetitive sequence was monitored for gain or loss of nucleotides. *DNMT1* mutants showed a higher rate of MSI compared to wild-type cells (Guo et al. 2004). Since expression of MMR components was unaltered, the consequences of *DNMT1* deficiency appeared to be direct rather than an indirect consequence of genomic hypomethylation or global changes in gene activity (Guo et al. 2004). In several independent studies, similar roles for DNMT1 were also reported (Gorbunova et al. 2004; Kim et al. 2004; Wang and James Shen 2004). In these reports, *DNMT1* deficiency resulted in a significant increase in MSI for both endogenous microsatellite loci (Wang and James Shen 2004) and transgenic slippage reporter constructs (Kim et al. 2004). In a genetic selection assay for trinucleotide repeat contractions, the DNA methyltransferase inhibiting drugs 5-aza-2'-deoxycytidine (5-azadC) and hydralazine further corroborated these findings—treatment with 5-azadC produced a 1,000-fold increase in the rate of MSI (Gorbunova et al. 2004). Although this selection assay was biased for the identification of contraction mutants, additional experiments utilizing human fibroblasts derived from myotonic dystrophy patients also showed large expansions of repeat tracts in response to 5-azadC treatment. These differing results hint at an underlying complexity in the involvement of DNMT1 in MSI.

One suggestion for the influence of DNMT1 on MSI is that microsatellite methylation provides a mechanism for

length stabilization by subsequent transcriptional repression of genes containing or proximal to microsatellites with methylated CpG repeats (Table 1). Transcription near or through microsatellites exposes single-stranded DNA to secondary structure formation that may be erroneously resolved through MMR (Lin et al. 2006; Lin and Wilson 2007). DNA methylation may stabilize these sequences by preventing them from being transcribed. In each of the above-mentioned analyses for MSI, non-CpG-containing repeat sequences were examined, thus DNA hypomethylation of the repetitive sequence itself is likely not the cause of MSI (Gorbunova et al. 2004; Kim et al. 2004; Wang et al. 2004). Furthermore, in one study, methylation levels of flanking DNA, which may also assist in stabilizing repeat sequences, did not differ between wild type and *DNMT1* mutant cells (Kim et al. 2004). In other analyses, however, CpG content was implicated in MSI. For CpG-containing human repeat sequences transfected into primate cells, DNA methylation stabilized CGG repeats during replication in instances where DNA was premethylated prior to transfection (Nichol Edamura et al. 2005). CpG methylation of these microsatellites significantly decreased the frequency of length change events and the magnitude of those changes (Nichol Edamura et al. 2005). CpG methylation of regions adjacent to microsatellites also contributed to repeat stabilization (Brock et al. 1999; Nichol and Pearson 2002). In one analysis which examined ten independent CAG-repeat-containing (non-CpG-containing) endogenous loci, differences in instability among the loci were noted (Brock et al. 1999). These differences were independent of microsatellite length but were correlated with flanking CpG content (Brock et al. 1999). Thus, in some cases, CpG content of microsatellites or neighboring cis-sequence does appear to play a role in stabilizing repeat length.

DNMT1 also influences transcriptional repression and MSI through chromatin remodeling (Table 1). Following DNA replication, DNMT1 mediates transcriptional repression and chromatin condensation through the histone deacetylases HDAC1 and HDAC2 (Fuks et al. 2000; Robertson et al. 2000; Rountree et al. 2000). Enhanced

trinucleotide repeat slippage in *DNMT1* mutants was correlated with an increase in H3 acetylation at the repeat, a mark of transcriptional permissiveness (Kim et al. 2004), and in a *Drosophila* model of repeat instability; reduced activity of the histone acetyltransferase CREB-binding protein (CBP) was responsible for CAG repeat expansion (Jung and Bonini 2007). Histone acetylation, however, cannot be entirely responsible for MSI as the HDAC inhibitor sodium butyrate produced only minimal destabilization of a trinucleotide repeat sequence (Gorbunova et al. 2004). Furthermore, contrary to arguments that transcriptional repression of microsatellite regions protects against MSI, overexpression of the HDAC SIRT1, which functions in gene silencing through chromatin remodeling, was linked to CpG island hypermethylation and high MSI in cancer cells (Nosho et al. 2009). This latter observation may allude to an indirect mechanism of global gene hypermethylation and gene silencing on MSI (as discussed below), and highlights the complexity of the links between epigenetic marks and MSI.

In addition to promoting microsatellite stability through transcriptional repression, another means by which DNMT1 promotes repeat stability is through a direct relationship between DNA methylation and MMR (Table 1). DNA methylation has been suggested to provide a mechanism for strand recognition following DNA replication—hemimethylated DNA generated by DNA replication may facilitate the MMR machinery in distinguishing the nascent strand from the template strand (Hare and Taylor 1985). The methyl CpG-binding protein MBD4/MED1 may provide the functional link between MMR and strand recognition. MBD4 co-localizes with foci of methylated DNA and was identified in a yeast 2-hybrid screen as interacting with the MMR component MLH1 (Bellacosa et al. 1999; Hendrich and Bird 1998). Although the mechanistic relationship between MBD4 and the MMR machinery remains undefined, MBD4 contains glycosylase repair activities with a preference for hemi-methylated CpG/CpT mismatches, thus providing a direct functional link between DNA methylation and MMR (Hendrich et al. 1999).

Yet another potential functional link between DNMT1 and MMR was revealed when DNMT1 was shown to interact with proliferating cell nuclear antigen (PCNA), the DNA polymerase processivity factor (Chuang et al. 1997). Loss of PCNA causes MSI, likely due to its function in DNA repair (Baida et al. 2003). PCNA promotes both MMR and nucleotide-excision repair through its interaction with the MMR factors MLH1 and MSH2, and PCNA rapidly recruits DNMT1 to sites of DNA repair through a direct interaction with DNMT1 (Mortusewicz et al. 2005; Nichols and Sancar 1992; Umar et al. 1996). Although the physical mechanism by which DNMT1 and these MMR proteins facilitate microsatellite stability is not known, the

association between these components and MSI is quite strong.

Microsatellite instability and human disease

Epigenetics, MMR, and cancer

An alternate, indirect mechanism in which genomic destabilization occurs is through DNA methylation-induced global changes in gene expression that alter transcriptional regulation of proteins responsible for repeat stabilization (Table 1), an effect that has been observed in cancer. In general, the genome of tumor cells is hypomethylated, particularly in regions of repetitive DNA. Widespread hypomethylation contributes to a hyper-mutation state in cancer, which may generate oncogenic mutations or loss of heterozygosity of tumor suppressor genes. Additionally, tumor cells also demonstrate sites of local hypermethylation, frequently at CpG islands that would, under normal circumstances, be hypomethylated (Robertson and Wolffe 2000). Site-specific CpG island hypermethylation affects genomic stability when it targets promoters of MMR genes. Along these lines, MSI is a common pathway to tumor development, and many MSI tumors contain mutations in MMR genes or epigenetic repression of MMR genes (Laghi et al. 2008).

An underlying methylation defect has been described in a subset of colorectal cancers in which promoter CpG islands are hypermethylated at high frequency, a condition termed the CpG island methylator phenotype (CIMP; Issa 2004; Toyota et al. 1999). One class of CIMP tumors displays MSI and concurrent inactivation of *MLH1* by promoter hypermethylation (Issa 2004). The first evidence of CIMP came from the finding that colorectal tumors lacking *MLH1* expression displayed *MLH1* promoter hypermethylation but no *MLH1* mutations (Kane et al. 1997), and subsequent reports have characterized MSI tumors as having a strong association with promoter hypermethylation compared to non-MSI tumors (Ahuja et al. 1997; Veigl et al. 1998). Moreover, experiments introducing a transgenic reporter gene into cancer cell lines revealed that expression of the reporter was associated with genetic instability: upon introduction of the transgene, cell lines deficient in MMR showed strong repression of the reporter gene compared to MMR proficient lines (Lengauer et al. 1997). The contrasting behavior of the transgene was linked to cell line-specific methylation differences that were negated by 5-azadC treatment, rescuing transgene expression (Lengauer et al. 1997). These reports are consistent with the idea that CIMP is associated with increased *DNMT1* expression or enhanced DNA methyltransferase activity (Teodoridis et al. 2008). Additional evidence from experiments in mice has further highlighted the importance

of DNA methyltransferase activity in CIMP tumors: mice deficient for *Mlh1* expression due to *Mlh1* promoter hypermethylation were less susceptible to intestinal tumor formation when *Dnmt1* function was also reduced (Trinh et al. 2002). Interestingly, although *Mlh1/Dnmt1*-deficient mice were less susceptible to intestinal tumorigenesis, they were more susceptible to lymphoid tumor development, a result linked to hypomethylation-induced genomic instability (Trinh et al. 2002). In *Dnmt1*-deficient mice, a similar phenotype was observed including genomic hypomethylation, increased lymphomagenesis, and marked genomic instability, particularly trisomy of chromosome 15 (Gaudet et al. 2003). Thus, DNA methylation is a double-edged sword in maintaining proper growth controls in that too much methylation enhances tumor-associated repression of repair genes, but too little methylation enhances genomic instability in repetitive regions that require a heterochromatic state for their stability. Obtaining a better understanding of the mechanisms of maintaining the proper balance and distribution of methyl marks in normal tissues will be critical for understanding how disruption of this balance contributes to cancer.

Other human disease associations with MSI

Trinucleotide repeat expansion is a hallmark of more than 20 human diseases including fragile X syndrome, Huntington's disease, and myotonic dystrophy (Mirkin 2007). These diseases are characterized by an “anticipation” phenomenon in which microsatellite expansion and disease severity worsen with each generation (Mirkin 2007). DNA methylation defects may directly contribute to the etiology of some repeat expansion diseases, because the developmental stages in which MSI occurs coincide with two key periods during which epigenetic reprogramming occurs (Gorbunova et al. 2004; Pearson 2003; Reik et al. 2001; Yoon et al. 2003). Moreover, DNA methylation was linked to stabilization of the CGG repeats whose expansion causes fragile X syndrome (Wohrle et al. 1998).

A key question is how do repeat expansions cause disease? One mechanism is through disruption of gene function: microsatellite expansions residing in exon or intron sequences disrupt the normal function of the gene product. Repeat expansions also produce local epigenetic changes. These expansions disrupt local chromatin structure and alter the expression of adjacent genes, an effect that has been extensively studied in myotonic dystrophy patients (Gatchel and Zoghbi 2005; Mirkin 2007). Myotonic dystrophy is associated with expansion of a CAG repeat within the 3'UTR of the *DMPK* gene. Repeat length is inversely correlated with expression of the flanking genes *DMPK* and *SIX5/DMAHP*, both of which have been implicated in the pathology of myotonic dystrophy (Klesert

et al. 2000, 1997; Thornton et al. 1997). Analysis of chromatin from skeletal muscle of individuals with myotonic dystrophy showed that the region surrounding the CAG expansion was protected from DNase digestion (Otten and Tapscott 1995), presumably due to a change in chromatin structure resulting in chromatin condensation (Otten and Tapscott 1995). The normal CAG locus was also shown to contain a region of H3K9 methylated heterochromatin embedded in a euchromatic H3K4 methylated region (Cho et al. 2005). The expanded repeat allele, in contrast, was associated with spreading of heterochromatin (and H3K9 methylation) into gene containing regions. Heterochromatin boundaries are normally limited by binding of the zinc-finger insulator protein CTCF; CTCF binding, however, is lost at the CAG boundaries in myotonic dystrophy (Cho et al. 2005; Filippova et al. 2001). Methylation of CTCF DNA binding sites prevents CTCF binding, which may, in turn, permit the spread of heterochromatin into surrounding euchromatic regions (Filippova et al. 2001).

Epigenetic modifications and DNA damage

In addition to repeat stabilization, DNA methylation and chromatin structure strongly influence other forms of genomic instability such as mutations and chromosomal rearrangements. For example, hypomethylation in *Dnmt1*-deficient murine ES cells produced a striking increase in gene mutations, deletions, and chromosomal deficiencies (Chen et al. 1998). Likewise, knockout of *DNMT1* and *DNMT3B* in a human colon cancer cell line caused large-scale, randomly occurring chromosomal translocations and aneuploidy (Karpf and Matsui 2005).

DNA methylation and homologous recombination

DNA methylation is well known to protect against unlicensed homologous recombination (HR), which is the basis for many mutational events. Following generation of a DNA double-strand break (DSB), HR is employed to form a synapse between homologous chromosomes, a circumstance that can generate gene conversions, insertions or deletions, or more extensive chromosomal loss. Transcription enhances HR frequency by exposing single-stranded DNA and allowing it to invade its homologous sequence, while DNA methylation-mediated transcriptional silencing inhibits HR (Dominguez-Bendala and McWhir 2004; Ikeda and Matsumoto 1979; Maloisel and Rossignol 1998). Several reports support the idea that DNMT1 represses HR. *Dnmt1* deficiency in mice resulted in elevated loss of heterozygosity due to mitotic recombination (Eden et al. 2003), *Dnmt1* deficiency in mouse ES cells

enhanced gene targeting by HR (Dominguez-Bendala and McWhir 2004), and embryonic carcinoma cells carrying integrated transgenic reporter constructs displayed increased recombination among transgenic sequences in response to depletion of DNA methylation by 5-azadC (McBurney et al. 2001). In a complementary study, hypermethylation of CpG islands within extrachromosomal fragments reduced the occurrence of deletions within and dimerizations between fragments, presumably due to reduced HR (Rizwana and Hahn 1999). Finally, DNMT1 and DNMT3 activity at repetitive pericentromeric and centromeric loci effectively limits mitotic recombination frequency and stabilizes centromeric repeats against length changes (Jaco et al. 2008).

Role of chromatin and DNA methyltransferases in DNA damage repair

In addition to the role of DNA methylation in prevention of HR, DNMT1 unexpectedly performs a role in DSB repair that appears to be independent of its maintenance methylation activity. DNMT1 has been shown to either assist in DSB detection or operate in the repair process itself. Induction of DSBs by irradiation or by 5-azadC shows an accumulation of DNMT1, its binding partners PCNA and MBD4, and the repair-associated factor MLH1 at foci of DSBs, and DNMT1 deficiency reduces the recruitment of DNA repair proteins to these foci (Mortusewicz et al. 2005; Palić et al. 2008; Ruzov et al. 2009). The well-established PCNA-DNMT1 interaction has recently been shown to be nonessential for DNMT1's maintenance methylation function (Schermelleh et al. 2007; Spada et al. 2007), and DNMT1 associates with chromatin independently of replication (Easwaran et al. 2004), suggesting a role for the PCNA-DNMT1 interaction separate from maintenance methylation. PCNA acts in multiple DNA repair processes including MMR and nucleotide excision repair, and thus DNMT1 may facilitate this function (Mortusewicz et al. 2005; Nichols and Sancar 1992; Umar et al. 1996). In summary, DNMT1 functions in the DNA damage response, perhaps through a methylation-independent role, highlighting an important and novel function for DNMT1 that deserves further examination.

DSB repair must operate in the context of complex higher-order chromatin structure and orchestrate restoration of chromatin and epigenetic marks at the site of DNA damage. Chromatin remodeling, like repair of the DNA itself, is critical for maintaining genomic integrity. Decondensation of chromatin in response to DNA damage may be required to loosen or open the chromatin structure and allow access for repair machinery (Ziv et al. 2006). Consistent with this notion, in *Saccharomyces cerevisiae*, HATs, including Gcn5 and Esa1, are actively recruited to sites of DSBs

presumably to assist in promoting open chromatin (Groth et al. 2007; Tamburini and Tyler 2005; van Attikum and Gasser 2005). This activity is only transient, and HDACs rapidly remove acetylation marks likely to prevent aberrant transcriptional activity in the newly repaired region (Tamburini and Tyler 2005). Chromatin remodeling following DNA damage is also mediated by the DNA damage checkpoint kinase CHK1. CHK1 is normally associated with chromatin in undamaged cells and specifically phosphorylates histone H3 at threonine 11 (H3T11) promoting acetylation of H3K9 and continued maintenance of cell cycle regulatory genes in a transcriptionally active state. Upon DNA damage, CHK1 dissociates from chromatin, and levels of phosphorylated H3T11 and acetylated H3K9 decline (Shimada et al. 2008; Smits et al. 2006). This deacetylation may function to repress transcription at the site of damage, providing protection for the decondensed chromatin against further vulnerability to mutations during the repair process.

Another critically important histone modification associated with DNA damage is phosphorylation of H2A, particularly at serine 129 and serine 1 (van Attikum and Gasser 2005). Recruitment of phosphorylated H2A.X (γ H2A.X), a variant of histone H2A, to sites of DSBs is a critical step in the repair process (Celeste et al. 2003, 2002). γ H2A.X-containing nucleosomes assemble along the regions bordering DSBs, as observed by immunofluorescence microscopy, and aid in the recruitment and/or maintenance of DNA break repair factors and histone modifiers at the site of damage (Rogakou et al. 2000, 1998; van Attikum and Gasser 2005). Notably, recruitment of γ H2A.X containing histones to sites of DSBs is also DNMT1 dependent (Palić et al. 2008). In addition, in both fertilized zygotes and cloned embryos, the occurrence of γ H2A.X-populated DSBs increased in a temporal pattern that coincided remarkably well with the period of active, DNA replication-independent demethylation during zygotic pronuclear development (Wossidlo et al. 2010). Although the mechanism underlying this connection is unclear, the association between these two events and the requirement for DNMT1 in the recruitment of γ H2A.X is consistent with a functional involvement of DNA methylation in DNA repair. It will be of great interest to determine the role of DNMT1 at sites of DNA damage and whether it is methylation dependent, as this may provide important information on how DNA methylation inhibitors exert their antitumor effects.

Chromatin modifications and DNA methylation in centromere function

Epigenetic determinants of centromere identity

A functional centromere is vital for guarding against chromosomal instability during cell division by maintaining

proper DNA segregation, averting chromosomal rearrangements, and preventing changes in ploidy. Cytologically, the centromere appears as a chromosomal constriction, but it actually consists of a molecularly unique and complex configuration of chromatin. Centromeric chromatin forms a densely compact structure that provides attachment sites for kinetochore proteins and spindle microtubules. The flanking pericentromeric chromatin recruits a high concentration of cohesin molecules providing the site for sister chromatid attachment (Allshire and Karpen 2008).

With every cell generation, each chromosome's centromere is habitually re-generated at the same location. Providing a single site for kinetochore attachment is necessary to ensure proper chromosomal segregation and stability, and numerous lines of evidence have established that centromere location/identity is determined epigenetically (Allshire and Karpen 2008; Amor and Choo 2002; Henikoff et al. 2001; Karpen and Allshire 1997; Sullivan et al. 2001). For example, cross species comparison shows that centromeric DNA sequences vary widely suggesting the lack of a single centromere recognition sequence. Likewise, human α -satellite repeat sequence within centromeric DNA is insufficient to generate a functional centromere, since chromosomes that contain two regions of centromeric α -satellite DNA, still possess only one functional kinetochore attachment region. Additionally, in the absence of a functional centromere, a neocentromere, which is a functional ectopic centromere, may form at alternate non-centromeric genetic loci that are devoid of α -satellite repeats (Amor and Choo 2002; Choo 2000). Having epigenetic centromeric determinants as opposed to sequence-specific determinants is advantageous because it provides a reliable mechanism for transmitting genetic material in the event of mutational damage to the centromere locus. Epigenetic determination also allows for adaptive karyotypic evolution and may provide an important mechanistic means for speciation (Henikoff et al. 2001). Thus, the epigenetically determined centromere provides a versatile system for consistent and faithful transmission of chromosomal material and for accommodation of karyotype alterations.

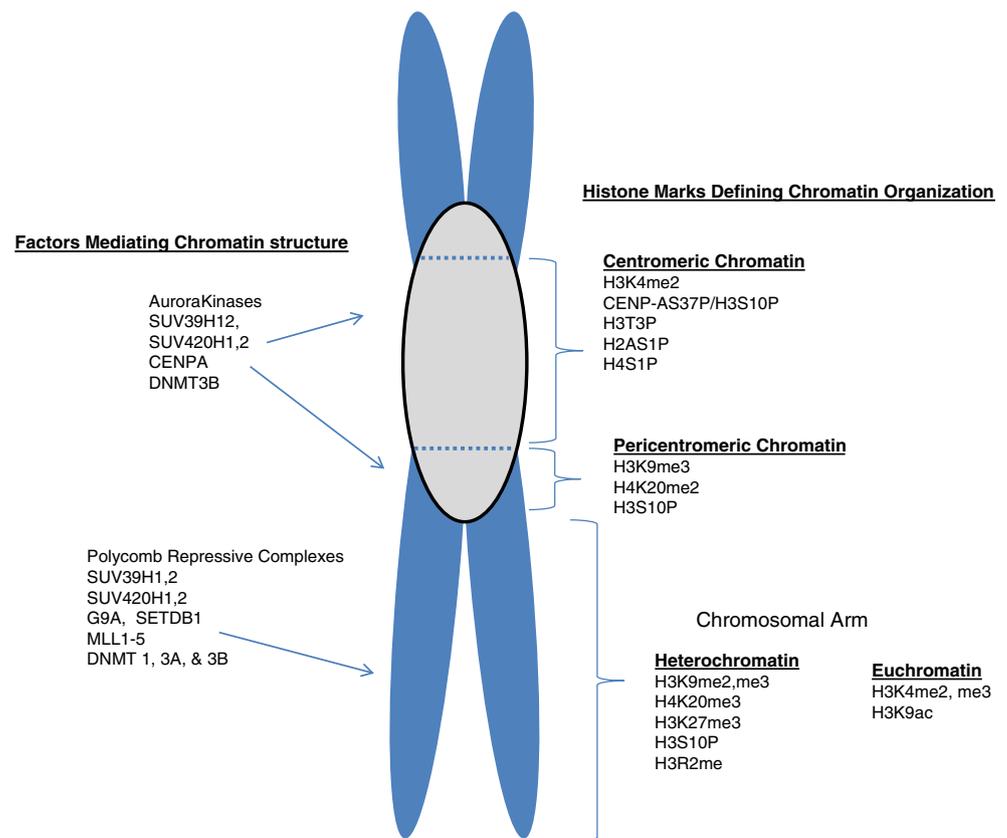
Human centromeres are characterized by several thousand kilobases of a 171-base pair AT-rich repeat referred to as α -satellite DNA (Amor and Choo 2002). Satellite DNA sequences typically do not possess similarity among chromosomes or across species, but their DNA-binding proteins are frequently conserved. One such important conserved centromeric chromatin protein is CENP-A, a centromere-specific core histone that substitutes for histone H3 in the nucleosome core (Palmer et al. 1987). Centromeric chromatin is composed of alternating blocks of H3 and CENP-A-containing nucleosomes (Blower et al. 2002). The structural significance of this CENP-A distribution is not known, but it may impart directionality onto the centromere structure that is necessary for kinetochore

attachment and for bi-directional, anaphase migration of sister chromatids (Allshire and Karpen 2008). Importantly, CENP-A is necessary and sufficient for recruitment of kinetochore proteins for mitosis (Palmer et al. 1991; Palmer et al. 1987). CENP-A deficiency disrupts kinetochore assembly and progression of mitosis, whereas CENP-A overexpression induces ectopic spreading of centromeric chromatin to non-centromeric locations and subsequent redistribution of kinetochore proteins (Allshire and Karpen 2008; Sullivan et al. 2001). Interestingly, CENP-A is recruited to DSBs, and cell survival following generation of DSBs is proportional to CENP-A activity (Zeitlin et al. 2009). Thus, CENP-A is multifaceted in its involvement in genomic maintenance and stability. H2A.Z, another histone variant replacing H2A in non-CENP-A-containing centromeric and pericentromeric nucleosomes, is also required for centromere structure and sister chromatid adhesion prior to chromosomal segregation (Greaves et al. 2007). The interplay between CENP-A and H2A.Z histone variants along with their unique arrangements of histone modifications (see below) appear to be key determinants in the organization and function of the centromere.

Histone modifications in centromere function and chromosomal condensation

Centromeric and pericentromeric chromatin contain unique and largely non-overlapping configurations of histone marks that are critical for centromere function (Fig. 1). Interestingly, despite the highly condensed nature of centromeric chromatin, its array of histone modifications is quite distinct from constitutive heterochromatin. Centromeric heterochromatin contains a characteristic combination of both euchromatic and heterochromatic marks (Lam et al. 2006; Sullivan and Karpen 2004). For example, condensed transcriptionally inert heterochromatin is defined by H3K9 di- and tri-methylation, but immunofluorescence studies showed that centromeric chromatin, despite being highly condensed, does not contain these marks (Sullivan and Karpen 2004). Surprisingly, CENP-A containing centromeric chromatin contains H3K4me₂, a mark associated with euchromatin and transcriptional permissiveness (Sullivan and Karpen 2004). However, other marks which confer an "open" euchromatic and transcriptionally active signature, such as H3K4me₃ and histone acetylation, are absent from centromeres (Sullivan and Karpen 2004). Pericentromeric heterochromatin, on the other hand, more closely resembles canonical heterochromatin. It is hypoacetylated and enriched for both H3K9me₂ and H3K9me₃; however, H3K9me₃ domains are located distally from the centromere, a configuration that may function to establish centromeric boundaries (Sullivan and Karpen 2004). Consistent with a functional role for H3K9 trimethylation in pericentromeric

Fig. 1 Chromosomal definition by histone modifications. Many molecules, including histone-modifying enzymes, histone variants, and DNA methyltransferases, some of which are depicted here on the *left*, establish the epigenetic marks and chromatin structure required for chromosomal organization and function. On the *right*, distinct combinations of histone modifications define chromatin regions including the centromere and pericentromere that organize and stabilize the chromosome as a whole and maintain genetic stability throughout repeated cell divisions



heterochromatin, the H3K9 methylases, SUV39H1 and SUV39H2, are critical for mitotic progression. SUV39H1 and SUV39H2 accumulate at centromeric domains during mitosis, and their loss, or the loss of H3K9 methylation, in pericentromeric regions is detrimental to chromosome segregation (McManus et al. 2006; Melcher et al. 2000; Peters et al. 2003, 2001; Rice et al. 2003). Also within pericentromeric domains, two additional histone methylases, SUV4-20H1 and SUV4-20H2, were shown to recognize H4K20 substrates for methylation, a modification necessary for chromosomal condensation and stability during mitosis (Oda et al. 2009; Schotta et al. 2004). SUV39H1/2 and SUV4-20H1/2 enzymes are also associated with recruitment and stabilization of heterochromatin protein 1 (HP1) to pericentromeric chromatin to mediate gene silencing and chromosomal condensation (Bannister et al. 2001; Lachner et al. 2001; Maison et al. 2002; Schotta et al. 2004; Souza et al. 2009). Suv39H1/2 activity is also important for CENP-A assembly into centromeric chromatin, since the fission yeast homolog of SUV39, Ctr4, along with HP1 and other heterochromatin associated factors, is necessary for loading CENP-A nucleosomes into centromeric regions (Folco et al. 2008). These studies emphasize how critical the distinctive patterns of centromeric and pericentromeric histone marks and their associated methylases are for the proper recruitment and stabilization of centromeric proteins. Global changes in histone methylation are also critical for whole

chromosomal condensation and centromere function during mitosis. Inhibition of overall histone methyltransferase activity just prior to prophase causes defects in chromosome alignment and segregation due to expansions and loosening of centromeric chromatin, unstable microtubule associations with kinetochores, and improper kinetochore protein localization (Heit et al. 2009).

Phosphorylation of serine and threonine residues, particularly on histones H1 and H3, is another modification critical for chromosomal condensation during cell division. Both histone H1 and H3 become phosphorylated upon entering mitosis and are dephosphorylated upon exiting mitosis (Gurley et al. 1978; Nowak and Corces 2004). The accumulation pattern of H3S10 phosphorylation is consistent with its requirement during mitosis given that it is initiated within pericentromeric regions during late G2 phase and is later removed during anaphase (Hendzel et al. 1997; Van Hooser et al. 1998; Wei et al. 1998, 1999). Absence of H3S10 phosphorylation yields incomplete chromosome condensation and defects in chromosomal segregation (Hsu et al. 2000; Wei et al. 1998, 1999). CENP-AS7 is phosphorylated similarly to H3S10, and the residues surrounding CENP-AS7 share similarity with those surrounding H3S10, suggesting functional conservation (Zeitlin et al. 2001a). Aurora kinases are responsible for H3S10 and CENP-AS7 phosphorylation, and inhibition of their activity results in loss of pericentromeric H3S10P

(Kunitoku et al. 2003; Monier et al. 2007). Aurora kinase phosphorylation of H3S10 recruits condensin to centromeric and pericentromeric regions to promote chromosomal condensation and rigidity (Giet and Glover 2001; Hsu et al. 2000; Ono et al. 2004; Zeitlin et al. 2001b). One of the critical factors recruited by H3S10P is HP1 α . Binding of the heterochromatic factor HP1 α at centromeric and pericentromeric marks throughout the cell cycle may be affected by the size of the H3S10P expression domain (Monier et al. 2007).

Phosphorylation of H2AS1 and H4S1 is also closely associated with mitosis and is similarly timed with the presence of H3S10P (Xu et al. 2009). Likewise, H3T3 shows patterns of mitosis-specific phosphorylation with particularly strong accumulation in centromeric regions during chromatin condensation (Dai et al. 2006, 2005; Eswaran et al. 2009; Polioudaki et al. 2004). H3T3 phosphorylation is generated by the kinase Haspin in regions devoid of H3K4 methylation, and its deficiency causes reduced sister chromatid cohesion and defects in metaphase chromosomal misalignment (Dai et al. 2006, 2005; Eswaran et al. 2009).

Another important chromatin configuration required for chromosomal condensation is the removal of histone acetylation via the HDACs (David et al. 2003). Acetylation is largely a mark of euchromatin and transcriptional permissiveness, and aberrant acetylation during mitosis reduces chromosome condensation and impairs sister chromatid separation (Cimini et al. 2003; Jeppesen et al. 1992). Inhibition of HDACs also causes loss of transcriptional repression in centromeric regions, chromosomal segregation defects, and chromosomal loss in fission yeast (Ekwall et al. 1997). Importantly, in fission yeast, the chromosomal condensation and segregation defects caused by HDAC inhibition persist even when HDAC activity is restored, suggesting the presence of an inherited mechanism for replicating mitotic deacetylation activity (Ekwall et al. 1997). In mammalian centromeres, histone deacetylase activity relies upon DNA methylation since DNMT1 is required for maintenance of histone deacetylation in pericentromeric domains (Xin et al. 2004).

DNA methylation in centromere function and kinetochore assembly

Centromeric and pericentromeric DNA is heavily methylated at CpG dinucleotides, a status that persists throughout the cell cycle and is important for centromere function (Monier et al. 2007; Wong et al. 2006). The requirement for this centromere-specific methylation is quite apparent in diseases characterized by chromosomal segregation defects, such as some cancers and ICF syndrome. These diseases, which demonstrate large-scale gain or loss of chromosomes, are often associated with loss of DNA methylation

at centromeric and/or pericentromeric loci (Gisselsson et al. 2005; Hansen et al. 1999; Lengauer et al. 1997; Wong et al. 2001). For example, a subset of hepatocellular carcinomas has been defined by particular chromosome 1 rearrangements stemming from a pericentromeric breakpoint, which was highly correlated with hypomethylation of pericentromeric satellite-2 CpG dinucleotides (Wong et al. 2001). The requirement for DNA methylation is also apparent in patients with ICF syndrome. ICF syndrome patients exhibit hypomethylation of heterochromatin, particularly of pericentromeric satellite-2 repeats within chromosomes 1, 9, and 16, resulting in decondensation of pericentromeric chromatin (Gisselsson et al. 2005; Hansen et al. 1999; Miniou et al. 1994, 1997). DNMT3B's PWWP domain is essential for methylation of these repeats, and significantly, the majority of ICF syndrome patients have mutations in *DNMT3B* that at least partially disrupt its methylation activity (Chen et al. 2004; Hansen et al. 1999; Xu et al. 1999).

The effects of DNA methylation and DNMT activity in pericentromeric and centromeric heterochromatin formation appear to be mediated through histone-modifying enzymes, heterochromatin factors, and centromeric proteins. Depletion of DNMT1 prevents accumulation of H3S10P foci, perhaps by affecting the subnuclear localization or the binding affinity of Aurora kinases to the centromere (Monier et al. 2007). DNMT3B also facilitates centromeric heterochromatin formation and chromosomal condensation through an association with condensin complexes (Geiman et al. 2004). DNMT3A and DNMT3B are recruited by H3K9me3-containing nucleosomes and SUV39H1 to pericentromeric domains, where they also interact with the heterochromatin factors HP1 β and HP1 α (Fuks et al. 2003; Geiman et al. 2004; Lehnertz et al. 2003).

DNA methylation and centromere function are further linked by the recent finding that DNMT3B interacts with the constitutive centromere protein CENP-C. CENP-C is an essential kinetochore protein that binds centromeric α -satellite DNA and is required for kinetochore assembly during mitosis (Kalitsis et al. 1998; Kwon et al. 2007; Politi et al. 2002). DNMT3B and CENP-C interact and become co-enriched at centromeric foci, reaching peak enrichment during metaphase (Gopalakrishnan et al. 2009). Importantly, this co-enrichment requires the mutual activity of both factors, since siRNA knockdown of either DNMT3B or CENP-C reduces the other's binding to centromeric and pericentromeric regions. This enrichment also facilitates DNMT3B's enzymatic activity, since CENP-C knockdown resulted in significant reduction in DNA methylation at both centromeric α -satellite DNA and pericentromeric satellite-2 sequence. Both CENP-C and DNMT3B are required for HP1 α recruitment to centromeric and pericentromeric regions, and their loss affects chromosomal

segregation and mitotic progression. Based on these findings, it is possible that loss of centromeric CENP-C and HP1 α recruitment in DNMT3B-deficient ICF patients contributes to the marked centromeric instability that is a hallmark of this disorder (Gopalakrishnan et al. 2009).

Importantly, DNA methylation within the centromere is not entirely ubiquitous and unrestrained, but it instead consists of specific non-methylated sites within extensively methylated regions. Non-methylated regions are particularly important for the kinetochore protein CENP-B. CENP-B, which interacts with both CENP-A and CENP-C, binds a specific 17-base pair sequence within α -satellite DNA and provides an important mechanism for centromere identity (Ando et al. 2002; Masumoto et al. 1989). Loss of CENP-B does not affect kinetochore assembly or function (Choo 2001; Hudson et al. 1998; Kapoor et al. 1998; Perez-Castro et al. 1998), but it instead functions to effectively limit multi-centromere formation and promote heterochromatin formation via SUV39H1 recruitment and DNA methylation (Okada et al. 2007). The centromeric pattern of methylation consisting of unmethylated CENP-B binding sites intermingled with extensively methylated regions is important for these functions since aberrant DNA methylation or 5-azadC treatment disrupts binding of CENP-B to these loci and alters its distribution (Miniou et al. 1997; Mitchell et al. 1996; Okada et al. 2007; Tanaka et al. 2005).

Interestingly, centromeric and pericentromeric DNA methylation is distinctive in that its function is not simply to promote widespread transcriptional silencing. Despite the dense DNA methylation in these regions, these genetic loci show signs of transcriptional activity (Lehnertz et al. 2003). Analysis of DNA methylation of neocentromeres showed that most CpGs are hypermethylated, except for small domains of hypomethylation corresponding to transcriptionally permissive sites (Wong et al. 2006). Furthermore, centromeres on human artificial chromosomes showed loss of function when transcription within the centromere was altered (Nakano et al. 2008). Either strong transcriptional repression or broad transcriptional activation caused loss of CENP-A, -B, and -C binding, loss of kinetochore assembly, and chromosomal missegregation (Nakano et al. 2008). Thus, studies using artificial chromosomes and neocentromeres suggest that a delicate balance between transcriptional activity and repression is required to maintain centromere functionality.

Conclusions

In summary, DNA methyltransferases affect chromosomal stability through DNA methylation, chromatin remodeling, and recruitment of heterochromatin specific factors. Genetic loss-of-function experiments involving the DNA methyltransferases have only just begun to elucidate their

pervasive effects on genomic stability and the crosstalk between DNA methylation and the histone code. Our understanding of how DNA methylation and the histone code affect each other to differentially establish functional, region-specific chromatin structure is still in its early stages, but it is increasingly evident that these epigenetic marks are critical for genomic stability and disruption of these mechanisms contributes to a growing number of human malignancies.

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Conflict of interest The authors have no conflicts to declare.

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