Chapter 24

Role of DNA Methylation in Genome Stability

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Chapter Outline

1. INTRODUCTION TO THE CELLULAR FUNCTIONS OF DNA METHYLATION

Genomic information is inscribed within the DNA sequences and additional chemical modifications embedded in the chromatin structure. The orders given by such information to the particular cell, neighboring cells, and even the entire organism based on the underlying signal transduction and crosstalk sustain all basic and normal functionalities, guiding survival, reproduction, death, and ultimately biological evolution. Disturbing genome stability by intrinsic and/or extrinsic factors could disrupt growth or developmental trajectory, as well as regular cellular behaviors, leading to abnormal or even detrimental consequences. Both genetic and epigenetic mechanisms introduce DNA sequence–dependent and –independent changes, resulting in detrimental consequences in a genomic content. Genetic mutations, deletions, insertions, translocations, and chromosomal aneuploidy are well-recognized consequences resulting from genomic instability. Remarkably, epigenetic mechanisms, namely DNA-methylation and histone modifications, are established and acknowledged as contributing factors for maintaining genome integrity through regulating these genetic events during different cellular processes. In this chapter, we focus on the genomic instability triggered by epigenetic changes with a specific emphasis on the role of DNA methylation.

1.1 DNA-Methylation Dynamics

DNA methylation is dynamic and subject to alterations. To date, the DNA methyltransferase family DNMT1, 3A, 3B, and 3L, and the DNA demethylases, ten–eleven translocation enzymes (TETs) and thymine DNA glycosylase (TDG), have been identified in animals (Fig. 24.1). A clear division of labor exists in each family. DNMT1 binds specifically to the hemimethylated DNA double helix and faithfully maintains methylation patterns in the newly synthesized DNA

Genome Stability. http://dx.doi.org/10.1016/B978-0-12-803309-8.00024-0

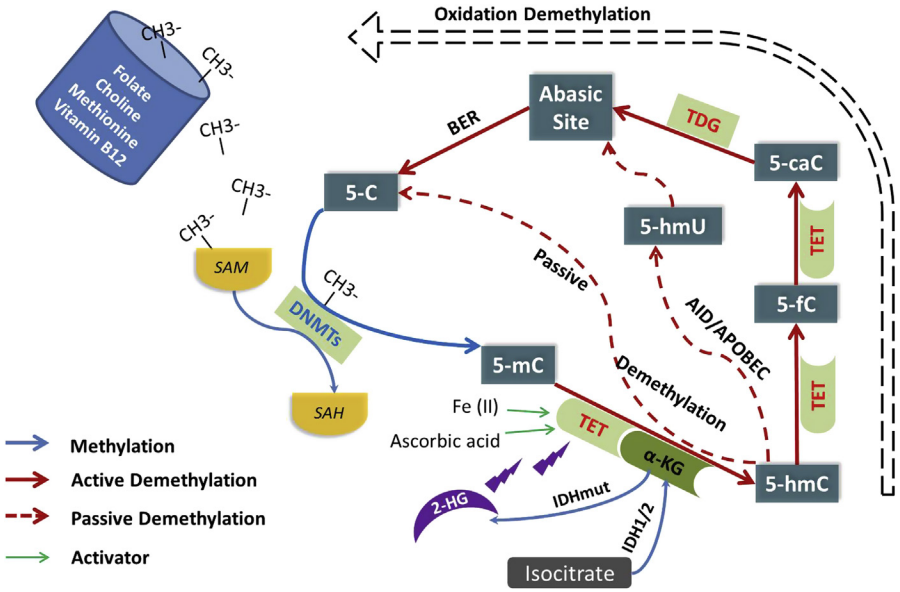

FIGURE 24.1 DNA-methylation metabolic cycle and associated essential nutrients. Methylation via DNMTs and active and passive demethylation via DNA methyltransferase (DNMT) and active and passive demethylation via ten–eleven translocation enzymes (TETs) and thymine DNA glycosylase (TDG) are depicted, along with natural resources of methyl donors, cofactors of TET, and effects of IDH1/2 mutations on demethylation enzymes. *Blue arrow*: S-adenosyl-L-methionine (SAM) assists DNMT activity by donating a methyl group to the DNA-methylation process, resulting in a methylated cytosine at position 5. *Red arrow*: Oxidation or active demethylation process takes place through the TET proteins, a dioxygenase protein family dependent on the availability of α-ketoglutarate and Fe^{2+}. TETs successively generate oxidized products 5-hydroxymethylC (5-hmC), 5-formylC (5-fC), and 5-carboxylC (5-caC). Highly oxidized 5-fC and 5-caC can be excised by TDG, forming an abasic site which can be repaired by base-excision repair (BER). *Red dash arrow*: Passive demethylation occurs in two possible pathways, one is through passive loss during replication, second is through AID/APOBEC-directed deamination process.

strand using the parental strand as a template [1]. This copy–paste process is essential for the inheritance of the biological information in the epigenomic structures to daughter cells during rapid cell proliferation. DNMT3A and DNMT3B are the de novo methyltransferases, capable of adding methyl-groups to the 5-position of unmodified cytosine, generating new patterns of DNA methylation [2]. DNMT3A and DNMT3B are extremely important in terms of establishing new DNA-methylation pattern during embryonic stem cell differentiation and tissue development [3]. Unlike stably expressed DNMT1, expression of DNMT3s is usually high in stem-like cells but reduced toward terminal differentiation. DNMT3L, lacking the C-terminal catalytic domain with the methyltransferase activity possessed by other DNMTs, mainly functions by influencing DNMT3A/3B activities to establish DNA-methylation markers [4]. De novo methylation drives the process of development and differentiation, and programs a cell with functional specificity [5]. It also creates dynamic DNA-methylation landscapes in response to intra- and extracellular signals, potentially contributing to environmental adaptation and evolutionary processes.

DNA demethylation is the process of removal or modification of a methyl (CH_3) group on DNA nucleotides. It can be achieved through both passive and active mechanisms. Passive demethylation could occur due to the absence of DNMT1 activity, with the newly synthesized DNA strands losing the methylation patterns such that upon several additional rounds of replication and division, this information will no longer be present in either strand. One of the examples of the passive loss of methylation is the passive demethylation upon inactivation of DNMT1 enzyme by 5-azacytidine [6]. Active DNA demethylation largely relies on the activity of TETs [7]. Unlike the relatively well-defined DNMTs, characterization of unique functions and possible redundancy of each TET are still underway. Oxidation of 5-methylcytosine (5-mC) takes place in a step-wise manner. In brief, TETs catalyze oxidation of an existing methyl group, yielding the first intermediate product 5-hydroxymethylC (5-hmC), which can be further oxidized into 5-formylC (5-fC) followed by 5-carboxylC (5-caC). Both 5-fC and 5-caC can be then replaced by an unmodified cytosine through TDG-mediated base-excision repair (BER). It worth noting that 5-hmC as well as the other two oxidation derivatives, 5-fC and 5-caC, are not recognized by DNMT1 during replication. Therefore, 5-hmC can be removed through active demethylation driven by TETs/TDG, or lost during replication by passive demethylation. Another proposed demethylation pathway involves a deamination process by cytidine deaminase (AID/APOBEC), which converts 5-hmC to 5-hydroxymethyluracil (5-hmU), generating an abasic site that can be removed by DNA glycosylase [8]. However, it is important to keep in mind that rather than just being

intermediate products of a demethylation pathway, emerging studies show that 5-hmC and 5-fC, although present at fairly low levels in the genome, are stable DNA marks and may play important roles such as regulating gene transcription and cell proliferation [9,10].

Through the methionine cycle, the level of methylation intermediates S-adenosyl-L-methionine (SAM) and S-adenosyl-homocysteine (SAH) sustain DNA-methylation reactions in the body [11]. Dietary factors, especially some micronutrients such as folate, methionine, and choline, are essential methyl donors to one-carbon metabolism [12]. Methyl donors target DNA methylation through regulating the substrate availability. Micronutrients such as iron and ascorbate are important cofactors of demethylation enzymes and have been shown to generate health concerns when they are not provided in sufficient quantity. Adding ascorbic acid to the cells with proficient expression of TETs is capable of inducing 5-mC oxidation, leading to a substantial loss of 5-mC and gain of 5-hmC, 5-fC, and 5-caC [13].

1.2 Transcriptional Regulation by DNA Methylation

DNA methylation at promoters and gene bodies regulates transcriptional activity in different ways. In animals, DNA methylation occurs primarily at cytosine in a cytosine–phosphate–guanine context (CpG). Although DNA methylation can also occur in the context of CHG and CHH (where H represents a nucleotide other than guanine), gene-regulatory functions of this form of DNA methylation are less clear. Genomic regions can thus be classified according to CpG density. The most CG-rich regions of the genome, CpG islands, are a frequently studied feature for DNA-methylation regulation. CpG islands are defined using a moving window of 500 bp with CG content more than 60%. CpG island shores, by definition, are regions 2000 bp upstream and downstream of a CpG island. Both CpG islands and CpG island shores have been confirmed to possess key regulatory functions in the genome. Hypermethylation of CpG island(s) in promoters usually leads to gene silencing, whereas hypomethylation permits active transcription. About 70% of mammalian gene promoters bear a CpG island, including those associated with housekeeping genes, developmental genes, tumor suppressors, and cell-cycle genes [14]. Aberrant hypermethylation at promoters of these genes, such as $p16^{INK4a}$, Rb, BRCA1, MLH1, and MGMT, is frequently observed in cancer or other diseases [14]. However, promoter methylation is not the only factor determining gene activity. For example, MGMT expression is often inhibited due to promoter hypermethylation in glioblastoma, but in tumors that have developed temozolomide-resistance, MGMT is reactivated even in the presence of promoter hypermethylation [15], suggesting alternative mechanisms exist to promote MGMT expression. In contrast to promoter methylation, gene body methylation is often associated with active transcription. As seen in 5-aza-2′-deoxycytosine (5-aza-2′-dC) treated HCT116 cells, loss of methylation at gene bodies correlates with transcriptional repression in a large set of genes, whereas DNMT3B-mediated methylation at these regions reestablishes gene expression [16]. Genome-wide DNA-methylation mapping via high throughput sequencing revealed that methylation patterns at CpG island shores display lineage- and tissue-specific patterns, and associated strongly with gene expression. This is supported by findings showing that disrupted DNA methylation occurs most frequently at CpG island shores in colon cancer concentrated at the regions with tissue-specific methylation, and results in a loss of tissue-specific epigenetic signatures, suggesting a role for DNA methylation in sustaining cell identity [17].

2. MULTIFACETED REGULATION OF GENOME STABILITY BY DNA METHYLATION

Disrupting DNA-methylation patterns established during cell growth and development leads to loss of function, cell-cycle arrest, and can even be favorable to disease development and transformation. This is not only because DNA methylation regulates transcriptional activities of cell-cycle genes, oncogenes, and tumor suppressor genes, but also because it influences mutation frequencies when inappropriate methylation occurs to noncoding regions and DNA damage–repair processes. In Section 2.1, we focus on the role of DNA methylation in restricting the expansion of repeat elements and preventing abnormal homologous recombination (HR). Next, DNA-repair mechanisms that prevent and/or correct genetic errors incurred during replication and chromosomal rearrangement will be linked to DNA methylation in Section 2.2. In Section 2.3, we will also discuss the contribution of DNA methylation in maintaining nucleosome and heterochromatin structure.

2.1 Chromosomal Rearrangement and Changes in Nucleic Acid Sequences

2.1.1 Instability of Repeat Elements

Noncoding regions occupy about 98.5% of the human genome and are an important contributor to genome/chromosome stability. Repeat elements comprise nearly half of these noncoding sequences. Repeat elements in the human genome are classified into two groups, interspersed repeats mainly comprised of transposable elements (TEs), and tandem repeats

ranging from a few bases to mega-bases [18]. Both classes of repeats are epigenetically modified, the status of which significantly contributes to genome stability and disease onset.

DNA-methylation mechanisms contribute to preserving stability of TEs (transposable elements) by silencing gene transcription, likely in a developmental stage–dependent manner. TEs are discrete mobile DNA segments capable of moving and integrating randomly within the genome. Depending on the nature of the element, transposition can be initiated by two different mechanisms, "cut and paste" and replicative transposition. DNA-only transposons are autonomous elements using a "cut and paste" mechanism initiated by transposes encoded within the transposon itself. The original repeat is directly relocated to the target site. In contrast, long terminal repeat (LTR) elements [19] and non-LTR elements are typical retrotransposons; they use replicative transposition requiring RNA synthesis and reverse transcription before the newly synthesized repeat sequence can be placed in the targeted site. Both long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) belong to the non-LTR family. Transposition induces genome instability in two ways. *First*, repeat element-directed recombination leads to intra- and interchromosomal rearrangement, dramatically increasing the frequency of deletions, duplications, and translocations. *Secondly*, transposition-associated mutations occur during RNA-based reverse transcription in autonomous retro-TEs, due to reduced processivity of the reverse transcriptase as compared to replicative polymerases. Both events are likely to have grave consequences on genome stability. In mouse embryos, transcription of intracisternal A-particle (IAP), a retrotransposon, is usually silenced due to a high degree of methylation in their LTR region. However in the absence of DNMT1, IAP transcript level increases by 50- to 100-fold, suggesting that DNMT1 plays an important role in maintaining the methylation level of IAPs [20]. In nondividing precursors of spermatogonial stem cells, deletion of DNMT3L disables the de novo methylation capacity at LTR and non-LTR retrotransposon elements of IAPs and thus enables active transcription [21]. Nonautonomous TEs (LINE and SINE) are critical components of heterochromatin enriched at regions flanking centromeres and telomeres [22]. DNA-methylation levels on these TEs are functionally relevant to the formation and stability of constitutive heterochromatin, delivering key messages to cell-cycle control and cell-fate decision markers. This is discussed further in Section 2.3.

Both DNMT1 protein and DNA methylation itself can stabilize tandem repeats. Tandem repeats are classified based on the size of the repeated sequence. A microsatellite comprises short tandem repeating units, usually less than 10 bp. Microsatellite instability (MSI) causes mutations through changes in length (expansion and contraction). It is therefore highly associated with hypermutation phenotypes in disease and contributes to lethal consequences in disorders like Huntington's, myotonic dystrophy, and a variety of cancers including hereditary nonpolyposis colorectal cancer (HNPCC). MSI is often a direct consequence of an impaired DNA mismatch repair (MMR) system. Accumulation of unrepaired DNA replication errors creates novel microsatellite fragments, or short tandem DNA repeats by definition, which are abundant in untranslated regions including introns. Mutations in DNMT1 result in increased instability of endogenous microsatellites and transgenic slippage reporter constructs without altering MMR components [23–25]. A novel function for *Dnmt1* in MMR was first assigned in genetic screening of *Blm*-deficient ES cells. This novel function of *Dnmt1* was confirmed in mouse ES cells in which cells with deficiency or homologous deletion of *Dnmt1* exhibited higher microsatellite slippage rate of a mononucleotide repeat carried by a reporter gene [24], as well as elevated frequencies of instability at endogenous microsatellite repeats [25]. More importantly, the flanking regions of mononucleotide repeats are always unmethylated regardless of *Dnmt1* expression level, suggesting that increased microsatellite slippage rate was not due to local DNA-methylation levels [24]. Other studies, however suggest that repeat stability is subject to DNA-methylation regulation at either local or adjacent regions. In human cells from myotonic dystrophy patients, inhibiting DNA methyltransferase through 5-aza-2′-dC treatment reproduces a similar consequence to that reported in the *Dnmt1*-deficient system, in which a 1000-fold increase in MSI was observed [26]. This effect could also be achieved through modifying CpG methylation of genes within or in the vicinity of microsatellites [27,28]. For example, expansion frequencies of CpG-free repeats, CAG.CTG, are highly affected by the CG content in the neighboring *cis*-sequence [26,28], suggesting methylation of CpGs at the flanking regions of microsatellite repeats also protects MSI from taking place. Trinucleotide repeats (TNRs) constitute a subset of microsatellites. Gain of methylation at CGG repeats artificially introduced into primate cells stabilizes these repeats [29]. Failure to maintain normal DNA-methylation patterns at repeat sequences during development contributes to the onset of genetic neurological disease. For example, fragile X syndrome (FXS) is caused by expansion of the CGG repeats at the fragile X mental retardation 1 (*FMR1*) gene on the X chromosome [30]. FMR1 mainly regulates dendritic protein synthesis, a class of proteins essential for synaptic strength. Normally, there are 30 CGG repeats at the 5′ untranslated region of FMR1. In patients with FXS, the number of repeats can be as high as 200 copies, resulting in hypermethylation of the entire repeat region and subsequent gene repression. A blockage in the AMPA-type glutamate receptor-signaling cascade arising from a lack of *FMR1* expression is primarily responsible for impaired learning and memory process [31]. Furthermore, $(CGG)_n$ repeat amplification is observed in the germline of male FXS patients carrying unmethylated repeats [32]. Another kind of TNR disorder, triggered by CAG repeat expansion, also accounts for a myriad of neurodegenerative disorders [33] such

as Huntington's disease, which is manifested by an increase of more than 35 consecutive CAG repeats on the gene encoding huntingtin [34]. Even though CAG repeats are devoid of CpG sites, studies show that DNA methylation of adjacent sequences is associated with CAG repeat stability. In a *Dnmt1*-knockout mouse model, intergenerational expansion of CAG repeats is observed at the spinocerebellar ataxia type 1 (*Sca1*) locus and is associated with aberrant DNA methylation at regions adjacent to the repeat tract [35]. Moreover, it appears that DNMT1 knockdown induces CAG repeat contraction through activating CAG repeat transcription [36]. This controversial observation suggests DNMT1 carries some complex functions remaining to be discovered. Interaction between DNMT1 and histone modifiers may also contribute to microsatellite stability. This is because DNMT1 interacts with histone deacetylases (HDAC1 and HDAC2) at microsatellites [37], promoting deacetylation so as to constrain the chromatin structure from being accessible to the transcriptional machinery. However, overexpressing HDACs does not necessarily reduce the frequency of MSI. In a human colorectal cancer (CRC) tissue survey, an inverse correlation was found between expression of *SIRT1*, a class III histone deacetylase, and incidence of MSI [38].

2.1.2 Chromosomal Recombination

DNA hypomethylation is generally associated with an elevated frequency of gene rearrangements and chromosomal translocations as a consequence of increased HR. HR occurs regularly during meiosis, naturally increasing the biological diversity within a species. It also occurs occasionally in somatic cells. The chances of HR increase significantly during transcription, when single-stranded DNA is exposed, spatially facilitating HR. It often occurs between DNA regions sharing extensive sequence identity (eg, sister chromatids) or highly similar (eg, two homologs) sequences. HR between repeated sequences leads to chromosome rearrangement, including deletions, duplications, and translocations of large DNA segments with disastrous consequences. Multiple lines of evidence showed that DNA methylation negatively affects HR in mammals [39–42]. These studies show that V(D)J recombination rate is significantly reduced by CpG methylation using minichromosome substrates [39], and that DNA hypomethylation at peri-centromeric satellite DNA is associated with increased rates of peri-centromeric chromosomal rearrangements [41]. Transcriptional silencing mediated by DNA methylation inhibits HR from taking place [43]. Studies in several mouse models, where genomic hypomethylation induced by a deficiency of *Dnmt1* resulted in an increase of HR [43] and loss of heterozygosity [44], suggest that *Dnmt1* contributes to repression of HR. Similarly, DNMT1 and DNMT3 recruitment to peri-centromeric and centromeric regions is believed to protect these loci against unlicensed HR [45]. Extensive DNA hypomethylation significantly increases mutation rates potentially through increasing the rate of mitotic chromosomal recombination. In ES cells carrying nullizygous *Dnmt1*, two specific genes, endogenous hypoxanthine phosphoribosyltransferase (*Hprt*) and a viral thymidine kinase (*tk*) transgene, show large increases in locus-specific deletions and mutations [40]. In 2011, a study using genome-wide sequencing identified that mutation rate varies across the genome [46] and is inversely correlated with DNA-methylation levels [47]. In particular, within CpGs sites, low (20–40%) to intermediate (40–60%) methylated CpG sites are prone to accumulate more mutations based on the density of single nucleotide polymorphisms (SNP) [47], again indicating that mutation rates are negatively correlated with methylation levels. The effects of aberrant DNA methylation on repeat elements are depicted in Fig. 24.2.

2.2 DNA-Damage Repair

DNA replication and chromosomal rearrangements are the most likely processes to yield mutations. The spontaneous error rate of mammalian DNA polymerase is about 10^{-5} to 10^{-6} per base pair, whereas the true mutation rate is only about 10^{-9} to 10^{-10}. This considerable reduction of final mutation rate is attributed to the polymerase proofreading system and DNA-damage repair. Depending on the type of DNA errors, different repair mechanisms will become active. Single nucleotide damage can be repaired by BER, nucleotide-excision repair (NER), MMR, and atypical modification of a specific nucleobase, such as 3-methyladenine and 8-oxoguanine, is corrected by BER through DNA glycosylase activity [48]. NER also responds when large and complex types of damage are found on DNA, such as intrastrand and DNA-protein cross-links, and bulky adduct formations [49]. Nucleotide misincorporation generated during DNA replication that escapes proofreading is resolved by MMR, as well as strand slippage- and recombination-resulted erroneous insertions and deletions at repeated DNA sequences (tandem repeats, microsatellites). MMR also corrects abnormally modified nucleotides including O^6-methylguanine (O^6-meG), 8-oxoguanine, and DNA adducts formed between DNA and carcinogenic chemicals through covalent bonds [50]. Repairing single nucleotide damage using an MMR mechanism requires the other strand as a template. DNA breaks can attack either one or both DNA strands. Single-strand breaks (SSB) result in discontinuity in one DNA strand and are often accompanied with loss of a single nucleotide. Filling the gap introduced by SSB requires the unbroken strand as template [51]. Double-strand breaks (DSBs) employ two mechanisms to repair, HR and nonhomologous end

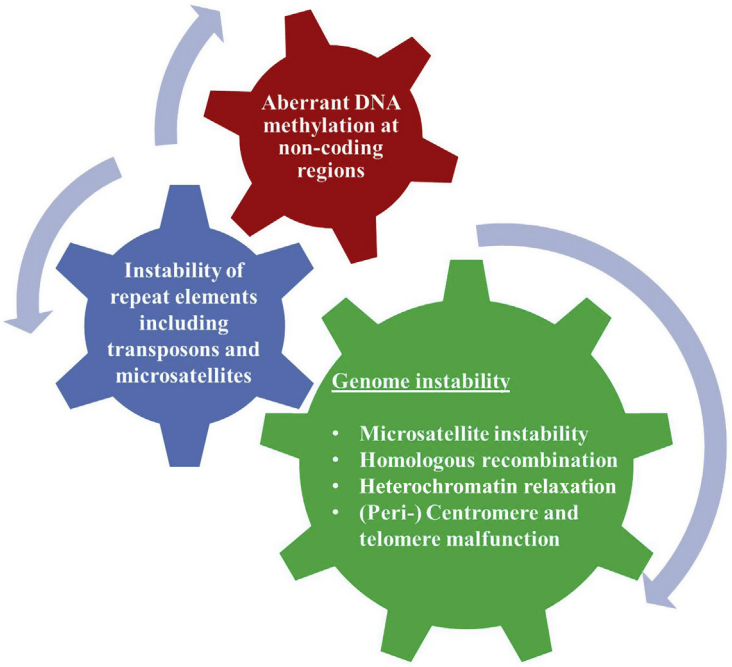

FIGURE 24.2 A chain reaction induced by DNA methylation at repeat elements. Aberrant DNA methylation at noncoding repeats destabilizes transposons and microsatellites, which result in microsatellite instability, increased rate of homologous recombination, heterochromatin structure change, and (peri-)centromere and telomere malfunction.

joining (NHEJ). HR takes advantage of the existence of a (nearly) identical sequence and uses it as a template for repair. NHEJ is mutagenic and therefore a less preferred mechanism as it usually results in point mutations and deletions of various size during repair [52]. The enzymes involved in each repairing process and their molecular functions are summarized in Table 24.1.

2.2.1 The Role of DNMT1 and DNA Methylation in DNA-Damage Repair

DNMT1 is an essential protein participating at the replication fork. Recruitment of DNMT1 to the replication fork requires interaction with proliferating cell nuclear antigen (PCNA), a cofactor of DNA polymerase delta (Polδ) and a component of DNA replication forks [53], and ubiquitin-like with PHD and ring finger domains 1 (UHRF1), a protein of unclear function that specifically recognizes hemi-methylated DNA and targets DNMT1 to such foci through a unique SET and ring-associated (SRA) domain [54]. UHRF1 and the complex formed by DNMT1 and G9a, euchromatic histone-lysine N-methyltransferase, colocalize with H3K9me2 at replication foci, enhancing the fidelity of DNA and histone methylation [55,56]. Depletion of DNMT1 at the replication fork leads to activation of checkpoint kinases 1 and 2 (CHK1 and CHK2, key effector kinases of the ATM/ATR-mediated DNA damage–response pathway), followed by degradation of cell division control protein 25a (CDC25a) and formation of γ H2A.X foci (H2A Histone family member X, a hallmark of DSBs), and eventually replication arrest [57]. This intra-S-phase replication arrest is not dependent on DNA demethylation as treating cells with 5-aza-dC, a nucleoside analogue trapping DNMT1 at the progressing replication fork, does not produce the same result [58]. Indeed, neither DNA demethylation by 5-aza-dC nor loss of catalytic activity of DNMT1 can stimulate a damage response similar to DNMT1 depletion [57]. Therefore, it appears that DNMT1 depletion triggers a protective mechanism to genome integrity through intra-S-phase replication arrest. It prevents global demethylation and epigenetic information loss by activating checkpoint pathways while being physically absent from the replication fork.

Accumulation of DNMT1 at DNA-damage sites and its association with MMR processes have been identified in a number of studies. The basic protein components of mammalian MMR are MutS (mutator S) α, MutSβ, MutLα, exonuclease 1 (EXO1), replication factor C (RFC), PCNA, replication protein A (RPA), DNA Polδ, and DNA ligase [59]. The MutS complex comprises a heterodimer of MSH2/MSH6 (MutS α) and MSH2/MSH3 (MutSβ), whereas the MutL complex consists of a heterodimer of MLH1/postmeiotic segregation increased 2 (PMS2) [60]. The principle of MMR resides in the nature of the DNA replication process, in which daughter strands should be faithfully synthesized using the parental sequence as the sole template. Therefore, upon receiving mismatching signals, three key actions are taken: *first*, recognition of the

TABLE 24.1 Key Enzymes of DNA-Repair Pathways

	DNA Glycosylase Scanning System				Endonuclease				DNA Polymerase	DNA Ligase	Reference
BER	UNG	OGG-1	NTHL1	NEIL1-3	APE1				Polβ	Lig1	[48]
	DNA Damage–Detection Complex				**Exonuclease Excision Complex**						
NER	XPC	XPG	RAD23B	ERCC6	XPA	XPG	RPA	ERCC1,3,4			[49]
	Mismatch-Recognition Complex				**Repair Machinery**		**Exonuclease**				
MMR	MutS	MutL			PCNA	RFC	EXO-1				[50]
	Tool Belt				**Approximation Process**						
NHEJ	Ku70	Ku80			Mre11–Rad50–Nbs1 (MRN)				–	Lig4	[52]
	DSB Processing		**Homologous Pairing and DNA Strand Invasion**					**Endonuclease**			
HR	MRN	Exo1	RAD51	RPA	BRAC2	XRCC2	XRCC3	Mus81–Eme1	Rev1, 3, 7	E3	[119]

mismatched base pair by MutSα complex and recruitment of MutLα, *secondly*, cleavage of the incorrectly placed nucleotide on the daughter strand by EXO1, and *lastly*, resynthesis of the damaged region by the PCNA/Polδ complex using the parental strand as a template [61]. A key premise of the MMR process is to distinguish between parental and daughter strands under the guidance of DNMT1. DNMT1 binds specifically to hemimethylated DNA during replication. MMR takes advantage of the hemimethylated state, identifies the parental strand, and then immediately digests the region containing the mismatched nucleotide (a short oligonucleotide spanning the mismatch site) on the new strand, allowing DNA polymerase to resynthesize the strand fragment [62]. In addition to being associated with PCNA at replication sties, DNMT1 also interacts with PCNA at DNA-damage sites [63] where MLH1 is also recruited [64], to methylate the new strand, demonstrating another aspect of DNMT1's role in MMR [63]. A protein–protein interaction between MLH1 and DNMT1 is possibly achieved through methyl-CpG binding domain 4 (MBD4), which binds MLH1 at its C-terminal glycosylase domain and DNMT1 via its N-terminal MBD domain [64]. Colocalization of DNMT1, MBD, and MLH1 occurs at heterochromatic regions and DNA-damage sites. In fact, DNMT1 deficiency impairs MMR function. Knockdown of DNMT1 in immortalized human fibroblasts yields resistance to the drug 6-thioguanine and a 10-fold increase of mutation rates at a CA_{17} microsatellite reporter gene, two hallmarks of MMR defects [65]. MMR defects in this study also appeared to be mediated by the reduction of steady-state protein levels of MSH2, MSH6, and PMS2. An important interaction between the MSH2/MSH6 heterodimer and DNMT1 was established in 2015 in a study of oxidation-induced DNA damage [66]. This study showed that oxidative damage triggered by hydrogen peroxide exposure reduces transcription of genes with promoter CpG islands. This repression is effectively blocked by knocking down MSH6 or DNMT1, suggesting accumulation of DNMT1 at the damaged site serves to prevent transcription from interfering with the repair process. An early study demonstrates that PCNA binds MSH6 and MSH3 at the replication fork during S phase [67], suggesting that accumulation of DNMT1 with MSH6/3 at the replication fork is likely through PCNA. In addition, DNMT1 contributes to DSB repair through interaction with both PCNA and ATR effector kinase CHK1. Immediately after laser microirradiation-induced DSBs, colocalization of DNMT1/PCNA/γH2A.X is observed at damage sites. The interaction between DNMT1 and PCNA or CHK1 is responsible for the recruitment of DNMT1 to the damage site, but is independent of its catalytic activity. This transient localization of DNMT1 to regions of DSBs modulates the rate of DSBs repair [68,69], again suggesting a methylation-independent role of DNMT1 in the DNA-repair process. MSI is a common mechanism for tumor development and can be driven by defective MMR. For example, knockdown of MMR components MSH2 or MSH3 inhibit contraction of CAG repeats, whereas depletion of MLH1 or PMS2 elevates contraction frequency [36]. Epigenetic silencing and mutations of MMR genes, including MLH1, MSH2, and PMS2, occur in many MSI tumors such as sporadic and hereditary colorectal and endometrial carcinomas [70]. This correlation between MMR and MSI has been brought into clinical application. Specially, the MSI phenotype is determined by MMR immunohistochemistry and is used to predict the risk of Lynch syndrome in patients with endometrial carcinomas [71]. These interactions between DNMT1 and other protein factors at replication fork and DNA-damaged site are illustrated in Fig. 24.3.

DNA damage could also be introduced by inappropriate DNA methylation. Exposure to alkylating agents, for example, results in the formation of O^6-meG, 1-methyladenine (1-meA), and 3-methylcytosine (3-meC). These aberrantly modified nucleotides form adducts, disrupt normal replication and transcription, and induce cell-cycle checkpoints and apoptosis [72]. Long-term accumulation of alkylation damage is prone to induce site-specific mutation (G to A) [73]. Direct reversal repair (DR) is involved to correct this type of DNA damage by employing two types of protein, O^6-methylguanine-DNA methyltransferase (MGMT or AGT) and the ALKBH family of Fe (II)/α-ketoglutarate-dependent dioxygenases (FeKGDs). Unlike BER or MMR, MGMT and ALKBH remove alkylation damage at DNA base-paring sites in a template-independent manner, and correct DNA base damage by directly accepting the methyl group [74]. The promoter of MGMT contains a CpG island, methylation of which usually remains low to ensure the proper expression of MGMT. Methylation of cytosine

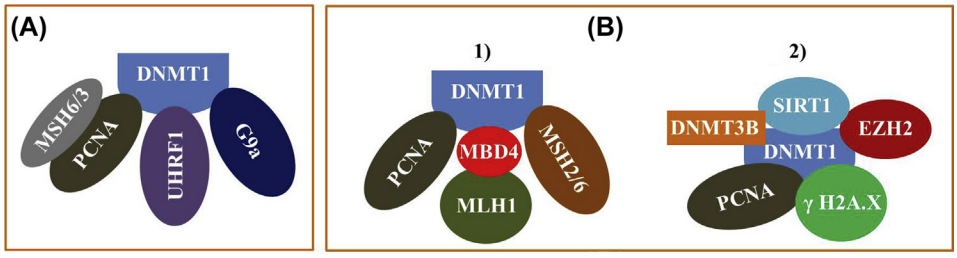

FIGURE 24.3 Protein–protein interactions between DNA methyltransferase (DNMT1) and DNA replication and repair proteins. Illustration of protein complex assembly at replication fork (A) and DNA-damaged sites (B). Accumulation of DNA mismatch repair (MMR) pathway-induced (1) and double-strand breaks (DSBs)-associated (2) DNA-repair protein is depicted, respectively.

is also mutagenic as it causes C to T transition mutations through deamination. The deamination product is mainly removed by thymine-DNA glycosylase, a key enzyme discussed in the context of the DNA-demethylation pathway.

Some interplay has been shown between HR-directed DNA damage repair, large DNA fragment exchange, and DNA methylation. HR serves as a means for repairing DSBs, resulting in gene conversion or loss of heterozygosity. Homologously recombined gene segments are often silenced through epigenetic mechanisms, involving DNA hypermethylation [75]. This event was induced at damaged site to repress local transcription from taking place [76,77], and achieved mainly through recruiting DNMT1 and the DNMT3s and introducing repressive histone modifications including H3K9me2/3 and H3K27me3 at the repair site [75,77]. Such epigenetic remodeling could either be transient or heritable, resulting in temporary or permanent gene silencing, respectively.

2.2.2 Transcriptional Regulation of DNA Damage–Repair Genes by DNA Methylation

MLH1 is an MMR protein that forms a complex with DNA-repair protein PMS2, and coordinates the other DNA-repair protein effectors to repair mismatches arising during DNA replication. Promoter hypermethylation of the MLH1 gene is highly associated with repressed expression, and is observed in many cancer types, including gastric cancer, nonsmall cell lung cancer, ovarian cancer, HNPCC, and CRC [60]. The frequency of MLH1 promoter hypermethylation however varies among cancer types and specimens, ranging from 1% to 66.9% in sporadic CRC, or from 0% to 21.4% in LS-CRC [78]. MLH1 promoter hypermethylation was observed in a subset of CRC with hypermethylation at a large number of CpG islands (termed CpG island methylator phenotype, or CIMP). In CIMP-positive CRC and gastric cancer, hypermethylation of MLH1 leads to a dysfunctional MMR pathway, resulting in an MSI phenotype [79,80]. This connection is supported by early evidence that MMR deficiency results in strong repression of a transgenic reporter gene through DNA hypermethylation [81].

Promoter methylation of the MGMT gene is a key factor determining the therapeutic efficacy in treating glioblastoma multiforme (GBM), one of most common and aggressive brain tumors. MGMT corrects the mutagenic DNA lesion O^6-meG in the DR pathway. During replication and transcription, O^6-meG mispairs with thymine. Thymine pairs with adenine in the next round of replication giving rise to permanent nucleotide alterations. Mutation or epigenetic silencing of MGMT is observed frequently in CRC [82]. Temozolomide (TMZ), an alkylating agent applied widely in chemotherapy, achieves better therapeutic effects when the MGMT promoter is hypermethylated [83,84]. This is because TMZ induces widespread N-7 or O-6 guanine methylation, which results in DNA damage accumulation and triggers cell death, but only when MGMT is not expressed. However, MGMT expression is not solely determined by promoter methylation. In GBM, long-term treatment of TMZ leads to drug resistance. In many cases of TMZ resistance, expression of MGMT is reactivated even with a hypermethylated promoter [15,85], suggesting that alternative gene-regulatory mechanisms exist.

As part of the BER pathway, TDG corrects G/T mismatches arising from the 5-mC deamination process. TDG interacts with deaminase AID and the damage response protein GADD45a (TDG is essential for active DNA demethylation by linked deamination BER). Promoter hypermethylation inhibits TDG expression. In multiple myeloma, epigenetic silencing of TDG contributes to genomic instability as it reduces DNA-repair efficiency [86]. Overexpression of TDG in cancer cell lines partially restores this DNA-repair pathway. Moreover, methylation-associated gene deregulation is found in many other DNA-repair genes, including XPC in bladder cancer, ERCC1 in GBM, and RAD23B in myeloma [60].

DNA methylation also regulates the transcription of genes involved in HR-directed DNA repair and NHEJ. HR promotes error-free repair by employing the sister chromatid as a template. Decreased rates of HR reduce DNA-repair efficiency, which is also carcinogenic. Cells deficient in breast cancer susceptibility gene 1 or 2 (BRCA1 or BRCA2) display reduced HR rate by at least sixfold in the presence of a DSB [87–89]. This is partially explained by the finding that both BRCA1 and BRAC2 interact with the RAD51 protein, which catalyzes the primary reaction in HR [90]. Epigenetic silencing of BRCA1 and BRCA2 genes by promoter hypermethylation is observed in breast cancer and several other cancer types [60]. In the NHEJ pathway, the XRCC5 gene that encodes the KU80 protein is also silenced by promoter hypermethylation, although this does not seem to be the only silencing mechanism in cancers like non-small-cell lung carcinoma where the gene is frequently down-regulated [91].

2.3 DNA Methylation and Heterochromatin Stability

2.3.1 Nucleosome Positioning and Packaging

Nucleosome structure and packaging are also influenced by DNA methylation. Studies reported during 2012–15 have used fluorescence resonance energy transfer (FRET) to monitor histone binding while modifying CpG methylation in a given DNA sequence. These studies revealed that CpG methylation of a DNA sequence tightened the association between double stranded DNA and core histone proteins, increased histone content within this region, and eventually expedited the

formation of more compact and rigid nucleosome structures [92,93]. Using the same method, another study showed that 5-hmC increases DNA binding to histones, but is more likely to keep the nucleosome in an open state for active transcription [94]. Interestingly, some contradictory results were raised from a current study using a nanopore-based force spectroscopy approach. In this method, the binding affinity between nucleosomal DNA and histone core proteins was examined by giving constant or time-varying force [95]. The result showed that nucleosome stability is more sequence dependent, rather than methylation dependent, as displacing DNA from the associated nucleosome required equal force regardless of methylation status.

2.3.2 Heterochromatin Instability

DNA-methylation patterns across the entire genome are responsible for establishing condensed heterochromatin domains or loose euchromatin domains. Two major types of heterochromatin are present in eukaryotic cells, *constitutive heterochromatin* that is enriched for tandemly repeated sequences and forms (peri-)centromeres or telomeres containing discrete satellite DNA, and *facultative heterochromatin* that comprises LINE-type repeats and silenced gene clusters that reversibly transition to euchromatin in the presence of developmental stage–dependent cellular cues [96]. Heterochromatin is tightly packed and localizes to the periphery of the nucleus. Maintenance of heterochromatin relies heavily on epigenetic landmarks, including nonrandom deposition of heterochromatin protein 1 (HP1) together with H3K9me3 and DNA methylation. HP1 keeps heterochromatin tightly packed and transcriptionally repressed. Interaction between HP1 and the nuclear membrane protein, lamin B receptor, contributes to heterochromatin localization. H3K9me3 recruits HP1 at constitutive heterochromatin [97], whereas H3K27me3 is mainly enriched at facultative heterochromatin. HP1 then attracts DNMT3B to the locus and stabilizes the region in heterochromatin by seeding DNA methylation. In addition, UHRF1, which facilitates DNMT1 recruitment, also specifically binds to H3K9me3 [98]. Methyl CpG binding protein 2 (MeCP2) recruits HDACs, which serve as an additional mechanism to maintain transcriptional inactivity and heterochromatin stability. In addition to its association with DNA replication sites during S phase, *Dnmt1* is also localized to constitutive heterochromatin during G2 and M phase [99]. Interestingly, this association exists independent of other heterochromatic marks like H3K9me3, suppressor of variegation 3-9 homolog 1 (Suv39H1) and HP1, suggesting a separate mechanism of establishing stable heterochromatin domains and maintenance of DNA methylation [99].

Heterochromatin at different chromosomal locations performs specific functions [100]. An inability to restrain heterochromatin territories by DNA-methylation or histone marks leads to malfunction and heterochromatin spreading [96]. During mitotic processes, chromosomal rearrangements may place an euchromatic region next to a heterochromatic region or remove the original boundaries protecting this euchromatic region, resulting in heterochromatin invasion into adjacent euchromatin and inactivation of gene clusters residing in this region. Alternatively, disrupting heterochromatin boundaries also leads to heterochromatin spreading, accompanied by DNA-methylation gains outside of the original regions [101]. Conversely, losing hallmarks of heterochromatin leads to deconstruction of heterochromatin structure. *Suv39h1/2*-deficient mouse embryonic fibroblasts exhibit severe chromosome mis-segregation and increased aneuploidy, suggesting a key role for *Suv39h* in maintaining genome stability [102]. Massive reduction in H3K9me3 and significant increase in transcription of peri-centromeric satellite 2 (Sat2) and centromeric α-satellite (α-Sat) are observed following loss of H3K9 methylation by inactivating Suv39H1. Both loss of H3K9me3 and transcriptional activation of satellite repeats are indicative of heterochromatin relaxation in this case [103]. Interestingly, DNA demethylation may induce a similar effect in that it is able to diminish H3K9me3 at the same loci [104]. Occupation of H3K9me3/HP1 usually prevents recruitment of the PRC1/2 complex. In the absence of DNA methylation at these loci, H3K27me3 level increases due to polycomb-group (PcG) protein binding. This colocalization pattern of H3K9me3 and H3K27me3 suggests that switching from constitutive to facultative heterochromatin requires an absence of DNA methylation.

Both centromeric and peri-centromeric heterochromatin serves as the structural basis for chromosome condensation and cohesion between sister chromatids, assisting proper segregation of mitotic chromosomes. Therefore, appropriate heterchromatinization at the peri-centromere satellites is a prerequisite for centromere function. DNA methylation is well known for its role in maintaining the integrity of peri-centromeric heterochromatin structure. For example, DNMT1 facilitates accumulation of H3S10P foci and Aurora-B targeting at peri-centromeres [105], whereas DNMT3B enables centromeric heterochromatin formation and chromosomal condensation [106]. Establishing DNA methylation at peri-centromeric heterochromatin also requires the Suv39H1/2 anchoring H3K9me3 marker [97]. DNMT3A and DNMT3B interact with HP1 via its chromodomain [97]. At peri-centromeric satellite repeats, coexistence of both DNA methylation and H3K9me3 has proved to be essential. *Suv39h1/2* double knockout in mouse cells profoundly reduced DNA methylation and *Dnmt1* binding at peri-centromeric heterochromatin [97]. An additional link between DNA methylation and centromere stability lies in the interaction between DNMTs and centromere proteins (CENPs). Both CENP-B [107] and CENP-C [108] are

important kinetochore proteins essential for ensuring proper kinetochore assembly during mitosis. CENP-B is crucial for centromere identity as it binds to unmethylated regions within the centromere to prevent the formation of multiple centromeres, while also promoting DNA methylation to maintain heterochromatin structure [107]. Colocalization of CENP-C and DNMT3B at centromeric regions is required for HP1 recruitment and kinetochore formation; loss of either mark results in a compromised association of the other to targeted sites, reduced DNA methylation, and impaired chromosomal segregation [109]. Some level of peri-centromeric repeat transcription has been shown to occur in most cells, but the underlying biological significance of these transcripts remains elusive. Although the exact role of DNA methylation in regulating this event is not yet clear, it is known that hypomethylation at this region in tumor cells results in transcriptional activation at peri-centromeric loci.

DNA methylation may also be responsible for maintaining telomere integrity through indirect regulation. Telomeres in most metazoans are comprised of a short DNA repeat sequence (5′-TTAGGG-3′) and are enriched for H3K9me3. Although these repeats do not appear to be directly affected by DNA methylation, an inverse relationship between sub-telomeric DNA methylation and telomere length and recombination was observed in a DNMT-deficient mouse model, which exhibited increased telomeric recombination and telomere-length changes [110]. A study reported in 2014 also suggested that DNA methylation at a subset of gene promoters is highly associated with telomere length in human leukocytes [111]. On the other hand, human telomerase gene expression can be activated following 5-aza-2′-dC treatment, suggesting that DNA methylation plays a role in regulating hTERT expression [112]. In addition, although positive correlations between telomere length and DNA methylation at LINE-1 and sub-telomeric regions in patients with dyskeratosis congenital were identified [113], this correlation was not stably observed across all research settings but rather was related to transcriptional and mutational landscapes [114]. For example, a study examining DNA methylation in human cancer cell lines showed no significant correlation between sub-telomeric methylation and telomere length [115]. Thus, whether and how DNA methylation affects telomeres is still controversial.

Immunodeficiency, centromere instability, facial anomalies (ICF) syndrome is a rare autosomal recessive immune disorder characterized by deficiency of serum immunoglobulin levels due to maturation blockage of naive B cells [116] and facial abnormalities. Different mutations have been mapped and are grouped into ICF subclasses, with type I (~50% of all cases) ICF carrying germline hypomorphic mutation in DNMT3B, type II (~30%) zinc-finger and BTB domain containing 24 (ZBTB24) mutations, type III cell division cycle associated 7 (CDCA7) mutations, and type IV lymphoid-specific helicase (LSH, or HELLS) mutations [117]. Hypomethylation at juxtacentromeric heterochromatin repeats accounts for the major pathogenic epigenetic mechanism that characterizes the genomic instability in ICF syndrome patients. In eukaryotes, integrity of centromeric heterochromatin is key for proper construction of cohesion and the kinetochore during mitosis. Centromeric regions and juxtacentromeric satellites are enriched with compact heterochromatin structures, methylation of which is usually maintained at a high level so as to maintain these DNA domains condensed, constrained, and silenced for transcription. Even though DNA methylation is not indispensable to heterochromatin formation, heavily methylated CpGs are believed to stabilize the heterochromatin structure. Extensive hypomethylation of constitutive heterochromatin regions results in loss of heterochromatin structure and consequential loss of mitosis-related functions. In ICF patients, classical satellite DNA is exclusively unmethylated in all tissue types, accompanied by chromosomal decondensation, frequent regional breakage, and rejoining taking place at satellite 2 regions of chromosomes 1 and 16, and satellite 3 regions of chromosome 9 [118]. It has been known that DNMT3B, through interaction with CENP-C, localizes specifically to the centromeric and peri-centromeric heterochromatin regions [109]. Mutation of DNMT3B in type I ICF leads to a hypomethylation phenotype and consequential abnormally arranged chromosome structure [109]. Although how mutations in ZBTB24, CDCA7, and HELLS contribute to the common epigenetic abnormalities and clinical manifestations in all ICF subclass remains to be answered, it is apparent that marked loss of methylation at (peri-)centromere regions is directly or indirectly attributed to these mutations.

3. CONCLUSIONS AND FUTURE DIRECTION

This chapter summarizes the essential functions of DNA methylation and DNA methyltransferases, especially DNMT1, in maintaining genome stability. DNA methylation at noncoding regions, including repeat sequences and heterochromatin regions such as centromeres and telomeres, inhibits spurious transcription and unlicensed HR, thus ensuring the proper functions of centromeres and telomeres at different cell stages. Disruption of these structures leads to mutations or genome rearrangements, which are monitored and repaired by a number of DNA-repair mechanisms. Proper performance of DNA-repair mechanisms requires both DNA methylation and DNMT1. DNA-methylation levels are involved in mediating expression of repair genes, whereas incorporating DNMT1 into replication forks and DNA damage sites through interacting with protein components of the DNA-repair machinery sustains the DNA-repair processes. Taken together, these results outline

the indispensable role of epigenetics, especially DNA methylation, in maintaining genome stability. Meanwhile, some intriguing questions are raised for future research: (1) The exact mechanisms of how and why DNMT1, independent of its methyltransferase function, regulates the DNA damage–repair processes are not fully defined. (2) The methylation modifications identified in 2009, 5-hmC, 5-fC, and 5-caC, although present at relatively low amount in the genome, regulate gene transcription and enhancer activities. However, less is known about their functional relevance to genetic stability and disease development. (3) As more evidence accumulates to define the relationships between DNA marks, chromatin modifiers, and their associated histone modifications, a cooperative epigenetic pattern may arise, that is particularly essential for sustaining heterochromatin integrity and DNA-repair functions. (4) Epigenetic mechanisms are an integral part of the etiologies for many types of cancer, as well as neurological and immune disorders. These diseases are often manifested by co-occurrence of genetic mutations and epigenetic modulations. Therefore, it is becoming increasingly important to assign "driver" and "passenger" roles to these events, so that effective therapeutic approaches can be implemented. This said, as of 2016, indirect connections between genetic mutations and epigenetic perturbations are constantly being discovered. For example, in type II, III, and IV ICF syndrome, mutations of the zinc-finger and BTB domain containing 24 (ZBTB24), cell division cycle associated 7 (CDCA7), and lymphoid-specific helicase (LSH, or HELLS) have been identified [117]. Given that the epigenetic abnormalities (marked loss of methylation at peri-centromeres and centromeres) are common to these subclasses, ICF syndrome provides a platform for discovering new epigenetic regulators. More importantly, in light of the fast-developing and increasingly applied high-throughput sequencing technologies, epigenetic research is no longer restricted to a limited number of gene loci; rather it becomes a genome-wide approach to understand the comprehensive gene-regulatory network in cell type–specific and developmental stage–dependent manners. Coupling genome-wide mapping for mutations, transcriptomes, copy number variations (CNVs), and SNPs, research connecting epigenetics to genetic stability is becoming broader, but also revealing previously unknown relationships between these pathways and the machinery that mediates them.

GLOSSARY

Chromosome rearrangement Abnormal structural change occurs to native chromosome resulting in deletions, duplications, inversions, and translocations.

Heterozygosity A genotype where two different alleles of a gene are present at the same locus of homologous chromosome.

Homologous recombination A process in which two similar or identical fragments of DNA exchange their genetic location.

Mismatch repair A strand-specific process that can recognize and repair errors arising from DNA replication- and recombination-induced insertions, deletions and nucleotide misincorporation, and certain other types of DNA damage.

Nullizygous A genome type in which both alleles lose function for the same gene but due to different means of mutation.

Retrotransposon A DNA transposon element transposes itself through a retroviral-like mechanism, in which an RNA template is transcribed and then reverse transcribed into a new DNA element for insertion.

Satellite DNA Large arrays of tandem repeats, mostly enriched at centromeric regions.

Tandem repeats A DNA fragment that contains multiple and adjacent copies of a sequence of two or nucleotides.

Transposase An enzyme that binds to the end of a transposon, cuts and then transports the transposon element to a different genetic location.

LIST OF ACRONYMS AND ABBREVIATIONS

5-aza-2′-dC 5-aza-2′-deoxycytidine

5-caC 5-Carboxylcytosine

5-fC 5-Formylcytosine

5-hmC 5-Hydroxymethylcytosine

5-mC 5-Methylcytosine

ATM/ATR Ataxia telangiectasia mutated, a serine/threonine protein kinase; ataxia telangiectasia and Rad3-related protein, a serine/threonine-protein kinase

CHK1/CHK2 Checkpoint kinase 1/2, two serine/threonine-specific protein kinases

CIMP CpG island methylator phenotype

CRC Colorectal cancer

DNMT DNA methyltransferase

DSBs Double-strand breaks

ERCC1 Excision repair cross-complementation group

FMR1 Fragile X mental retardation one

FXS Fragile X syndrome

GBM Glioblastoma multiforme

H3K9me2/3 Di-/trimethylated histone H3-lysine nine

H3K27me3 Trimethylated histone H3-lysine 27
HDAC Histone deacetylase
HR Homologous recombination
IAP Intracisternal A-particle
ICF Immunodeficiency, centromeric instability, and facial anomalies
LINE Long interspersed nuclear element
LTR Long terminal repeat
MBD Methyl CpG-binding domain
MGMT O^6-alkylguanine DNA alkyltransferase
MLH1 MutL homolog 1
MMR DNA mismatch repair
MSI Microsatellite instability
PCNA Proliferating cell nuclear antigen
PMS2 Postmeiotic segregation increased 2
SAM S-adenosylmethionine
SINE Short interspersed nuclear element
TDG Thymine-DNA glycosylase
TEs Transposon elements
TET Ten–eleven translocation enzymes
TMZ Temozolomide
TNR Trinucleotide repeats
XPC Xeroderma pigmentosum, complementation group C

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