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Review

DNA Methylation, Its Mediators and Genome Integrity

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Abstract

DNA methylation regulates many cellular processes, including embryonic development, transcription, chromatin structure, X-chromosome inactivation, genomic imprinting and chromosome stability. DNA methyltransferases establish and maintain the presence of 5-methylcytosine (5mC), and ten-eleven translocation cytosine dioxygenases (TETs) oxidise 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be removed by base excision repair (BER) proteins. Multiple forms of DNA methylation are recognised by methyl-CpG binding proteins (MeCPs), which play vital roles in chromatin-based transcriptional regulation, DNA repair and replication. Accordingly, defects in DNA methylation and its mediators may cause silencing of tumour suppressor genes and misregulation of multiple cell cycles, DNA repair and chromosome stability genes, and hence contribute to genome instability in various human diseases, including cancer. Thus, understanding functional genetic mutations and aberrant expression of these DNA methylation mediators is critical to deciphering the crosstalk between concurrent genetic and epigenetic alterations in specific cancer types and to the development of new therapeutic strategies.

Key words: DNA methylation; DNA methyltransferases; methyl-CpG binding proteins; DNA glycosylases; BRCA1; genome instability.

Introduction

The presence of 5-methylcytosine (5mC) was first demonstrated in tubercle bacillus DNA in 1925 [1] and in calf thymus DNA two decades later [2]. Although biological functions of this cytosine modification remained uncharacterised for decades, in 1975, two studies demonstrated important roles of 5mC as an epigenetic modification that influences gene expression [3, 4] and highlighted the significance of the 'fifth nucleotide' in eukaryotic biology [5]. DNA methylation is now widely recognised as a typical epigenetic mark because it satisfies the stringent criterion of an epigenetic system that is mitotically and meiotically heritable as redefined from Waddington [6] by Riggs in 1996 [7]. In prokaryotes, methylation at both adenine (A) and cytosine (C) residues contributes to host restriction systems and protects the cell from foreign genetic materials such as bacterial and viral genomes [8]. In contrast, DNA methylation in multicellular eukaryotes occurs predominantly but not exclusively at cytosine residues within CpG dinucleotides [9]. In vertebrates, DNA methylation is a major form of epigenetic modification and is regulated during development to control tissue and differentiation states [10]. Moreover, DNA methylation patterns are altered in cancers and in embryos produced by somatic cell nuclear transfer [11, 12]. These changes contribute significantly to the molecular pathology of these disease states [12-14].

In this review, we introduce various forms of DNA methylation in terms of distributions and transcriptional mechanisms in mammals. We summarise recent advances in the understanding of mediators of

DNA methylation and demethylation, including DNA methyltransferases (DNMTs), methyl-CpG binding proteins (MeCPs), ten-eleven translocation cytosine dioxygenases (TETs) and base excision repair (BER) DNA glycosylases. Aberrant transcription of cell cycle, DNA repair and chromosome stability genes are associated with promoter hypermethylation of corresponding transcriptional start sites (TSSs) with CpG islands (CGIs) in human cancers and were recently linked with germ-line and somatic mutations in their gene bodies without CGIs. Moreover, a major role of DNA methylation in modelling chromatin structure, which generally regulates gene expression states and thus the accessibility of DNA for damage, is discussed. We emphasise the current understanding of genetic and epigenomic alterations involving DNA methylation mediators in human cancers and discuss their potential influence on carcinogenesis by providing selective growth advantages for tumour transformation and aggression.

Multiple Forms and Genomic Distribution of Cytosine DNA Methylation

Multiple forms of DNA methylation have been identified in mammals, including 5mC, the recently discovered 5-hydroxymethylcytosine (5hmC) and the ensuing oxidation products 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) [15-17]. The major epigenetic modification 5mC and its hydroxylated derivative 5hmC are relatively stable and abundant in mammalian genomes [18] (Fig. 1A). In contrast, 5fC and 5caC are extremely rare and can be transiently removed by thymine DNA glycosylase (TDG) and are therefore speculated as active DNA demethylation intermediates [17, 19, 20]. DNMTs produce 5mC by covalently adding a methyl group to the 5-position of the cytosine ring, predominantly occurring at CpG dinucleotides in somatic cells [21, 22]. Mounting associations of 5mC with gene silencing indicate important roles in normal mammalian genomic imprinting, X-chromosome inactivation, repetitive element suppression and lineage-specific gene expression regulation [13, 23, 24]. However, non-CpG methylation also occurs with high frequency in mouse and human embryonic stem (ES) cells and induced pluripotent stem cells [21, 22]. The 5hmC intermediate was recently discovered as a second modification in vertebrate DNA and is formed by addition of a hydroxy group to 5mC by TETs [16, 25] (Fig. 1A). These enzymes are enriched in Purkinje neurons and ES cells [15, 16], and because 5hmC is more stable than its oxidation products 5fC and 5caC, the hydroxymethyl group is likely to have biological properties and may be an epigenetic mark [26, 27]. Because 5mC and 5hmC are only distinguishable in experiments using 5hmC specific antibodies [28, 29], recent developments have been aimed at resolving 5hmC sites in the genome [30, 31]. Nonetheless, it is accepted that 5mC is the most prominent modification in vertebrate DNA in the majority of mammalian tissues [32].

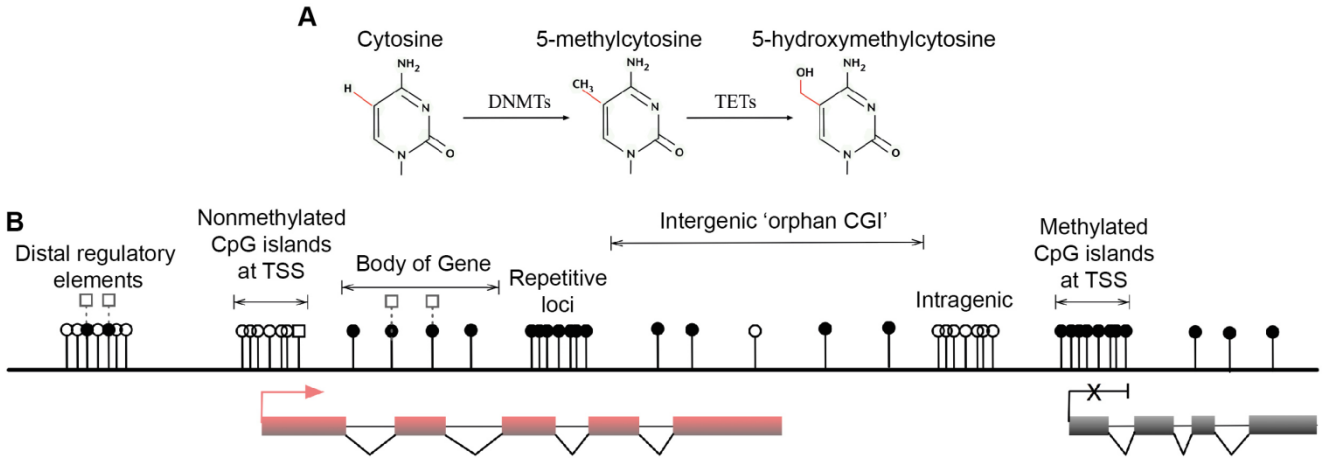

Figure 1. Major forms and distribution of DNA methylation. (**A**) The three major forms of cytosine bases in mammalian DNA. The 5-position of cytosine is covalently methylated by DNA cytosine methyltransferases (DNMTs) with the presence of co-factor S-adenosyl methionine (SAM). The resulting 5-methylcytosine (5mC) is mostly found on CpG dinucleotides in somatic cells. 5-hydroxymethylcytosine (5hmC) is formed by methylation and subsequent hydroxylation and is mediated by the ten-eleven translocation cytosine dioxygenases (TETs). (**B**) Distribution of CpG dinucleotides in mammalian genomes. In vertebrate genomes, CpG dinucleotides are generally highly methylated, whereas CpG islands (CGIs) that are associated with gene promoters have exceptional global unmethylated patterns. Exceptions include CGIs on inactive X-chromosomes in female cells, where CGIs are hypermethylated. In addition to canonical CGIs located at annotated transcription start sites (TSSs), orphan CGIs of unknown function are found within gene bodies (intragenic) and between annotated genes (intergenic). Unmethylated CGIs at 5' ends of multiple genes are positively correlated with transcriptional activity (active, left), whereas a small number of genes are hypermethylated at their promoter CGIs and are repressed in specific cell types (inactive, right). Gene bodies are often methylated with higher DNA methylation at exons than introns, and 5hmC is present at expressed gene bodies and are the proposed 5mC oxidation products of TET enzymes (labelled white squares at body of gene). White circles, nonmethylated CpGs; black circles, methylated CpGs; white squares, hydroxylmethylated CpGs; red boxes, active and transcribed exons; black boxes, inactive and silenced exons; transcriptional states of these genes are represented by the red arrow (active) and the black cross (inactive).

Genome-wide studies demonstrated direct regulation of a number of developmental genes by DNA methylation [33, 34]. Most (70%–80%) mammalian CpG sites are methylated, and highly methylated sequences are found in satellite DNAs, repetitive elements, gene bodies and non-repetitive intergenic DNA [35]. However, CpG islands (CGIs), the CpG-rich regions that are located in more than half of the promoters of mammalian genes (approximately 60% in human genes) have exceptional global unmethylated patterns [36-38]. Unmethylated CGIs in the vicinity of promoter regions are normally associated with tissue-specific expression of corresponding genes in early embryos and ensuing somatic cells. However, these CGIs become largely *de novo* methylated during X-chromosome inactivation, resulting in legitimate gene silencing on the inactivated chromosome, which is required for dosage compensation [39]. Moreover, although only 100–200 annotated CGIs were thought to be present in somatic cells [33], a recent study identified approximately 23,000 and 25,500 non-annotated 'orphan' CGIs in mouse and human genomes, respectively [14] (Fig. 1B. Top). A large number of these under-represented CGIs were found in the proximity of annotated TSSs of constitutively expressed genes and at intergenic regions and gene bodies [14]. These orphan CGIs may act as promoters but are often methylated during development [14], suggesting limited transcriptional activity. Associations of DNA methylation at these regulatory sequences and transcriptional repression in somatic cells are well established, and the potential long-term impact on the stability of gene expression profiles is widely accepted [40]. In general, DNA methylation of GC-rich gene promoter regions is associated with gene repression, whereas transcribed genes usually correlate with low DNA methylation levels around TSSs and high levels in the gene body [21, 41, 42] (Fig. 1B. Lower left and right). In pathophysiological states, both global DNA hypomethylation at repetitive and satellite regions of the genome and site-specific hypermethylation of CGIs at promoters of tumour suppressor genes are associated with whole genome instability, a hallmark of human cancers [43-47].

Mediators of Cytosine DNA Methylation

DNA Methyltransferases, the Canonical 'Writers'

The methyltransferase enzymes DNMT1, DNMT3A and DNMT3B harmonise in the establishment and maintenance of DNA methylation patterns in mammals (Fig. 2A and Table 1). DNMT3A and DNMT3B are *de novo* methyltransferases that target cytosines of previously unmethylated CpG dinucleotides. These enzymes have an equal preference for hemimethylated and unmethylated DNA, which are essential for their roles in *de novo* methylation of the genome during development and for newly integrated retroviral sequences [48, 49]. Following the first wave of genome-wide demethylation in the preimplantation embryo, Dnmt3a and Dnmt3b are highly expressed at implantation and re-establish a bimodal methylation pattern that effects more than 80% of the genome [48], whereas most CGIs are protected by unknown mechanisms and therefore remain unmodified [41]. Genetic and functional analyses indicate that Dnmt3a and Dnmt3b have non-overlapping functions during development with different phenotypes and lethality stages [48], suggesting that each enzyme has regional specificity that reflects their respective N-terminal domains. Accordingly, Dnmt3a is necessary for maternal imprinting at differentially methylated regions, and Dnmt3b is required for methylation of pericentromeric repeats and CGIs on inactive X-chromosomes [50]. Established DNA methylation patterns are stably preserved over cell divisions by DNA methyltransferase-1 (DNMT1), which is known as a maintenance enzyme that guards existing methylated sites through its preference for hemimethylated DNA [51]. Dnmt1 is particularly present at high concentrations in dividing cells [51], localising perpetually to replication foci [52]. Dnmt1 operates with its methylation co-factor UHRF1 (Np95) in protein complexes that constitute an enzymatic platform, providing a maintenance methyltransferase function for CpG methylation [53-55]. In addition to its methyltransferase activity, DNMT1 has a proliferating cell nuclear antigen-interacting domain, replication-targeting region, cysteine-rich Zn^{2+}-binding domain, nuclear localisation signal and polybromo-1 like protein domain [56, 57]. It also contains an N-terminal region that is associated with various chromatin-associated proteins, including *de novo* methyltransferases, histone modifying enzymes and MeCPs. Among DNMTs, DNMT2 shows weak methyltransferase activity *in vitro*, and its depletion has little impact on global CpG methylation levels and no discernible effects on developmental phenotypes [51]. Moreover, although DNMT3L (DNMT3-like) is catalytically inactive, it is highly expressed in germ and ES cells and acts as an obligatory cofactor for *de novo* methyltransferase in ES cells [58]. Dnmt3L stimulates the methyltransferase activity of Dnmt3a or Dnmt3b through physical interaction [59-62]. Crystallographic analyses of Dnmt3a and Dnmt3L indicate that these interactions may be mediated by a heterotetrameric complex formation [63], which may prevent Dnmt3a oligomerisation and heterochromatic localisation [64]. A recent study

showed that DNMT3L is a positive regulator of DNA methylation at gene bodies of housekeeping genes and a negative regulator of DNA methylation at promoters of bivalent genes in mouse ES cells, suggesting a dual role in ES cell differentiation [65].

Methyl-CpG Binding Proteins, the Invited 'Interpreters'

The 'written' methylation marks at CpG dinucleotides can be specifically recognised by various MeCPs, which may in principle 'read' the established methylated DNA sequences and recruit histone-modifying complexes to regulate higher order chromatin structure, stabilise patterns of gene expression and maintain genome integrity [66]. Among the three major characterised families of mammalian MeCPs (Fig. 2B and Table 1), methyl-CpG binding domain (MBD) proteins, including MeCP2, MBD1, MBD2 and MBD4, but not MBD3, specifically recognise 5-methyl-CpG (5meCpG) dinucleotides via novel MBD domain. The second KAISO family comprises three structurally unrelated zinc-finger proteins KAISO/ZBTB33, ZBTB4 and ZBTB38, and KAISO proteins have been shown to bind to methylated DNA through zinc-finger motifs. The third family includes two 5meCpG-binding ubiquitin-like proteins UHRF1 and UHRF2, which recognise methylated DNA via RING finger-associated (SRA) domains.

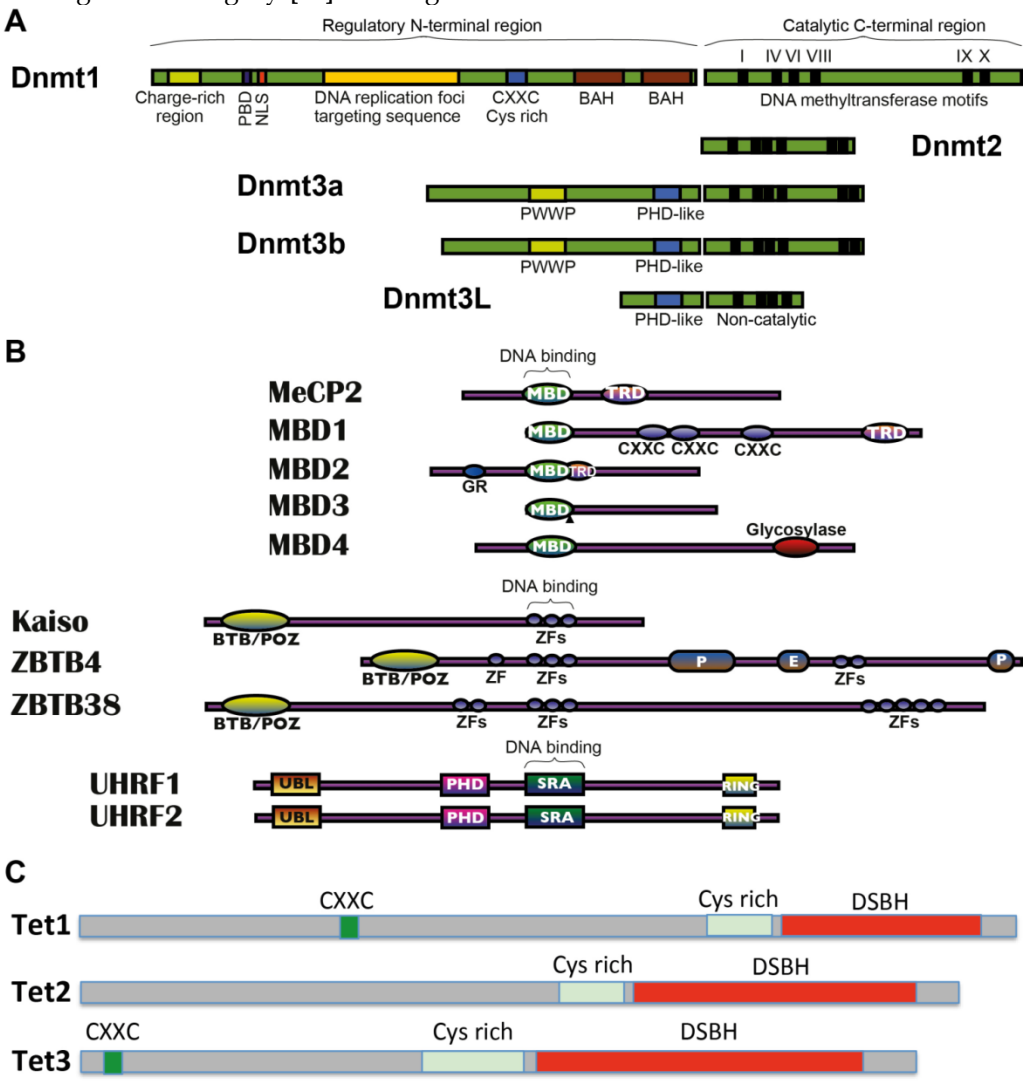

Figure 2. Mediators of DNA methylation machinery. (**A**) Domain structures of mammalian DNA methyltransferases (DNMTs). Functional domains in the N-terminal regions of DNMTs are shown and the conserved motifs in the C-terminal region are labelled. In the N-terminal region, the sub-domains include a proliferating cell nuclear antigen binding site (PBD), nuclear localisation signal region (NLS), plant homeo domain (PHD) like domain and PWWP domain (highly conserved proline–tryptophan–tryptophan–proline motif that is involved in protein–protein interactions) and bromo-adjacent homology domains (BAH). N- and C-terminal domains are linked by Gly-Lys dipeptides. Highly conserved C-terminal methyltransferase motifs are shown as thick black lines (indicated as I–X). (**B**) Domain structures of methyl-CpG binding proteins (MeCPs). Three families of characterised mammalian MeCPs include (1) the methyl-CpG binding domain proteins (MBDs) MBD1, MBD2, MBD3, MBD4 and MeCP2. (2) the structurally unrelated methyl-CpG binding zinc-finger proteins of the Kaiso family KAISO/ZBTB33, ZBTB4 and ZBTB38 and (3) the methyl-CpG binding SRA domain proteins of the UHRF family UHRF1 and its homologue UHRF2. Labelled sub-domains include MBD, methyl-CpG binding domain; TRD, trans-repressor domain; GR, E, P, amino acid repeats; BTB/POZ, broad complex, tramtrack, and bric à brac domains; ZF, zinc finger motifs; UBL, ubiquitin-like motif; PHD, Plant homeodomain and SRA, SET and Ring-associated domain. DNA binding regions are indicated. (**C**) Domain structures of ten-eleven translocation methylcytosine dioxygenases (TETs). Schematic representation of conserved domains of mouse Tet proteins is shown, including a double-stranded–helix (DSBH) fold (all Tets), cysteine-rich (Cys-rich) domain (all Tets) and CXXC zinc fingers (Tet1 and Tet3).

Table 1. Functions and specificities of human DNA methyltransferases and methyl-CpG binding proteins

Gene symbol	Gene name	Function	DNA Specificity	Human Tumours [67, 68]	Mouse knock-out phenotype (MGI database)
Human DNA methyltransferases					
DNMT1	DNA methyltransferase 1	Maintenance DNA methyltransferase	Hemimethylated DNA	Gene body Mutations in colorectal cancers [69]. Overexpression in leukaemia [70], gliomas [71], non-small cell lung [72], pancreatic [73], gastric [74], hepatocellular [75] and breast cancers [76].	Stunted and delayed development and embryonic lethal by E9.5 [77]. Lack of appropriate genomic imprinting [78].
DNMT2	DNA methyltransferase 2	low DNA methyltransferase activity; RNA (tRNA) methyltransferase activity	Cytosine 38 of transfer RNA^{Asp}	Reduced expression in hepatocellular [79], colorectal and stomach cancers [80].	No phenotype observed [81]. Direct data from MGI reported a decreased proportion of natural killer cells in the peripheral blood.
DNMT3A	DNA methyltransferase 3A	*De novo* DNA methyltransferase	Equal preference for unmethylated and hemimethylated DNA	Mutations in AML [82, 83]. Overexpression in gastric [84], hepatocellular [75], pancreatic [85] and colon cancers [86].	Normal development at birth but become runted and die around four weeks of age [48].
DNMT3B	DNA methyltransferase 3B	*De novo* DNA methyltransferase	Equal preference for unmethylated and hemimethylated DNA	Mutations in ICF syndrome [87], SNP in lung cancer [88]. Overexpression in leukaemia [70], glioblastoma [71], gastric [84], hepatocellular [75], colon [86, 89], prostate [90] and breast cancers [91]	Embryonic lethal before E9.5 with growth retardation and rostral neural tube defects. Slight under-methylation of endogenous viral DNA, substantial demethylation of minor satellite DNA [48].
DNMT3L	DNA methyltransferase 3L	Cofactor; required for de novo methyltransferase activity in ES cells	/	Potential biomarker for cervical cancer [92] and embryonal carcinoma [93].	Lack appropriate methylation of the maternal allele and cause azoospermia in homozygous males [94]; heterozygous progeny of homozygous females die by midgestation [95].
Human methyl-CpG binding proteins					
MeCP2	Methyl CpG binding protein 2	Methyl-CpG binding; transcriptional repression	Symmetric 5meCpG; A/T-rich sequence adjacent to 5meCpG for efficient DNA binding shown by in vitro assays	/	Female mice homozygous or male mice hemizygous for a null allele and heterozygous mice exhibit neural, Rett syndrome-like symptoms [96, 97].
MBD1	Methyl-CpG binding domain protein 1	Methyl-CpG binding; transcriptional repression	5meCpG within TCGCA and TGCGCA sequence context	Mutations in lung and breast cancers [67].	Defects in adult hippocampal neurogenesis and function, impaired spatial learning, reduced neuronal differentiation and increased genomic instability [98].
MBD2	Methyl-CpG binding domain protein 2	Methyl-CpG binding; transcriptional repression and activation	5meCpG in a single orientation	Mutations in lung and breast cancers [67].	Viable and fertile; defective maternal nurturing behaviour, decreased tumourigenesis [99].
MBD3	Methyl-CpG binding domain protein 3	Component of Mi-2/NuRD complex; transcriptional repression	/	Decreased expression in gastric carcinogenesis [100].	Embryonic lethal because of failure in differentiation of pluripotent cells [99, 101].
MBD4	Methyl-CpG binding domain protein 4	Methyl-CpG binding; BER DNA glycosylase; apoptosis; transcriptional repression	Symmetric 5meCpG	Mutations in colon, endometrial and pancreatic cancers [102, 103].	Viable and fertile. Increased rate of C to T mutation at CpG dinucleotides [104, 105].
KAISO/ ZBTB33	KAISO/ zinc finger and BTB domain containing 33	Methyl-CpG binding; transcriptional repression; Wnt signalling suppression	two 5meCpG motifs in close proximity preferably in tandem	Indicator of aggressive prostate cancers, associate with high grade and triple-negative invasive breast cancer, poor prognosis in non-small-cell lung cancer.	viable, fertile and overtly normal with no detectable changes; reduced tumourigenesis [106].
ZBTB4	Zinc finger and BTB domain containing 4	Methyl-CpG binding; transcriptional repression	Unmethylated consensus sequence $C^{C}/_{T}GCCATC$; strong binding specificity to single methylated CpG in a surrounding nucleotide-specific manner	Possible prognostic marker and potential therapeutic target for breast cancer survival [107].	/
ZBTB38	Zinc finger and BTB domain containing 38	Methyl-CpG binding; transcriptional repression	Bind to a single methylated CpG	/	/
UHRF1	Ubiquitin-like with PHD and ring finger domains 1	Cofactor for the DNA methylation maintenance; transcriptional regulation; E3 ubiquitin ligase activity for histone H3	hemimethylated DNA	Overexpression in a variety of human cancers including those of the breast, liver, lung, bladder, which often correlate with a poor outcome [108-110].	Embryonic lethal in gestation showing growth retardation and various malformations because of essential defects of global and local DNA methylation [111].
UHRF2	Ubiquitin-like with PHD and ring finger domains 2	Ubiquitin E3 ligase; SUMO E3 ligase; specific recogniser of 5hmC	hemimethylated DNA	Possible predictor of survival and potential therapeutic target in colon cancer [112].	/

Although all MeCPs share binding specificity for symmetrical 5meCpG dinucleotides, some have additional binding preferences. For example, the MBD domain of MBD1 recognises 5meCpG dinucleotides more efficiently within TCGCA and TGCGCA sequences [113], whereas MBD2 in chickens (96% homology to human MBD2) binds to 5meCpG dinucleotides in a single orientation, suggesting that additional sequences outside of the 5meCpG dinucleotides are necessary for MBD2 specificity [114]. In addition, efficient DNA binding of MeCP2 requires an A/T-rich sequence adjacent to 5meCpG dinucleotides, and *in vitro* assays showed the involvement of few amino acids upstream of the MBD domain of MeCP2 [115]. In the second MeCP family, the zinc finger domain of KAISO proteins was shown to have a binding preference for two 5meCpG motifs in close proximity, preferably in tandem [116-118]. The third UHRF family recognises 5meCpG using a distinct base-flipping mechanism [119, 120], which was demonstrated for the SRA domain of UHRF1 that recognises and binds hemi-methylated DNA and acts in conjunction with DNMT1 to maintain DNA methylation [120, 121]. UHRF2 has an SRA domain with 75% homology to UHRF1 and was shown to have a similar binding preference to hemimethylated DNA *in vitro* [122]. Moreover, a recent study showed the presence of a weak but specific affinity for hemi- and fully-hydroxymethylated DNA [123].

DNA methylation *per se* alters transcriptional binding sites to prevent transcriptional activation and the binding of transcriptional factors such as E2F or CREB [124, 125]. In addition, MeCPs can act as 'interpreters' that specifically recognise 5meCpG marks and subsequently recruit various chromatin modifiers to establish a repressive chromatin environment [126-129]. Almost all MeCPs have been demonstrated to associate with transcriptional repressors, implying an additional layer of regulation between DNA methylation and transcription. As an example, MeCP2 associates with co-repressor complexes such as Sin3A and NCoR via its trans-repressor domain (TRD) and induces strong global transcriptional repression [127, 130]. Moreover, MBD3 is an essential subunit of the Mi-2/NuRD chromatin remodelling complex, which was shown to function as a transcriptional repressor complex both *in vivo* and in cell culture assays [66].

Cytosine Dioxygenase TETs and Its 5mC Product 5hmC

The TET enzyme family comprises three cytosine dioxygenases, including TET1 and its two dioxygenase paralogues TET2 and TET3 (Fig. 2C and Table 1). These proteins are Fe(II)/α-ketoglutarate (αKG)-dependent dioxygenases of the AlkB family, which share a double-stranded β–helix (DSBH) catalytic domain. The DSBH domain of two AlkB family J-binding proteins JBP1 and JBP2 oxidise the 5-methyl group on thymine (T) to 5-hydroxymethyluracil (5hmU) [131]. JBP protein homology and similarities of 5mC and T at the 5-position of the cytosine ring suggested that TET proteins convert 5mC to 5hmC [25]. Accordingly, TET1 was shown to generate 5hmC from 5mC by oxidation of the methyl group in a Fe(II)/αKG-dependent manner [16], and all TET proteins were subsequently shown to catalyse stepwise conversions of 5mC to 5hmC, 5fC and 5caC *in vitro* and *in vivo* [19, 132, 133]. C-terminal catalytic domains of TET proteins contain an indispensable cysteine-rich region adjacent to their DSBH domain [134]. In addition, TET1 and TET3 carry a cysteine–X–X–cysteine (CXXC) domain at the N-terminus that strongly binds to unmethylated DNA [135].

Among the three 5mC oxidation forms, 5hmC is the most stable, but its presence significantly varies among tissues [136]. Recent studies suggested that 5hmC is predominantly enriched in the vicinity of transcription factor binding sites, including distal-regulatory elements and gene bodies of highly expressed genes, and is less abundant at gene promoter regions [30, 137] (Fig. 1B). In principle, 5hmC distribution at these gene regulatory loci may be associated with stable regulation of gene expression across the whole genome. As noted above, 5mC modification, particularly at promoter regions, is associated with gene repression. Accordingly, substitution of 5mC residues with 5hmC may inhibit recruitment of the classic 5mC interpreter MeCPs and undermine subsequent transcriptional repression activities. In support of this idea, *in vitro* studies showed that MeCP2 has a markedly reduced binding affinity for 5hmC in contrast to its strong binding affinity for 5mC [138-140]. The less bound MeCP2 may release the associated histone modifying enzymes that produce overall histone deacetylation or specific lysine methylation [126-128], deregulating the repressive transcription environment. Another study demonstrated an equal binding affinity of MeCP2 for both 5mC and 5hmC [141], perhaps owing to the experimental differences in terms of selected DNA probes and/or truncated protein. Thus, further investigations are required to test the possibility that conversion of 5mC to 5hmC inhibits potentially the binding to MeCPs. The relative abundance of 5hmC in brain and ES cells suggested that it has additional DNA demethylation-independent functions that are 'read' via specific 5hmC interpreters [142]. Several 5hmC candidate 'readers' have recently been identified, including UHRF2, UHRF1 and MeCP2 [139, 141-143], although the last two have been shown to bind to

5mC-containing DNA with equal or greater affinity *in vitro* [139]. A recent crystallographic study with structural and biochemical analyses confirmed that UHRF2 specifically recognises 5hmC with approximately 1.5 and 3.2 times affinity in hemi- and fully-hydroxymethylated DNA, respectively, than in hemimethylated controls [123]. Moreover, the conformation of a phenylalanine within the SRA domain of UHRF2 was shown to optimise the preferential binding pocket for 5hmC [123]. This study also used electrophoretic mobility shift assays to examine the binding affinity of SRA domain and a longer version of UHRF2 to DNA probes containing 15 CpGs with unmodified or modified cytosines and showed that UHRF2 preferentially binds 5hmC over 5mC modified probes via its SRA domain. Because numerous proteins containing zinc-fingers or SRA domains are encoded in mammalian genomes, novel classes of 5hmC-binding proteins with distinct binding specificities may be identified in future studies [123, 142]. As for 5fC and 5caC, two recent studies used proteomics approaches to identify proteins that show a strong binding preference for 5hmC and further oxidation products [142, 144]. Consistent with the hypothesis that separate functions exist for these unmodified or modified cytosines as epigenetic marks, both studies reported a large number of proteins that can be specifically recruited by 5fC and 5caC probes *in vitro*, including transcription regulators and DNA glycosylases, supporting that 5hmC and further oxidation products 5fC and 5caC may function separately.

BER Glycosylases Act as Mediators of 5mC Enzymatic Removal

Dynamic changes of 5mC patterns in developmental and pathophysiological states suggested that active removal of 5mC occurs in an enzyme-dependent manner [145]. Current lines of investigation favour mechanisms that involve conversion of 5mC to the deamination products T and 5hmU [146, 147] and oxidation products 5fC and 5caC [19, 133, 147], which can be subsequently excised and repaired by DNA glycosylases following activation of BER pathways [145]. In principle, DNA glycosylases, such as TDG and MBD4, initiate BER repair by cleavage of the glycosidic bond between the 5mC base and deoxyribose [148]. This activity uses a base-flipping mechanism and generates abasic apurinic/apyrimidinic (AP) sites, which can be efficiently recognised and processed by AP endonucleases, DNA polymerases and DNA ligases. Subsequently, deoxyribose is removed and replaced by a nonmethylated cytosine, which restores the original DNA sequence. At least four bifunctional DNA glycosylases have been identified in plants, including ROS1, DEMETER (DME), and the DME-like proteins 2 and 3, which remove 5mC from both CpG and non-CpG regions [145]. In 1995, vertebrate DNA glycosylases were shown to actively reverse DNA methylation, and weak 5mC glycosylase activity was observed in chicken embryo nuclear extracts. A later study demonstrated the involvement of the chicken homologue of human TDG [145]. Whereas precise global and gene-specific mechanisms of DNA demethylation have not been demonstrated in mammals, recent evidence strongly suggested the mammalian DNA glycosylases TDG and MBD4 activate the BER repair pathway to remove intermediate residues that are generated in proposed pathways of oxidation, deamination or both [35, 145, 149]. Nonetheless, direct removal of 5mC by these mammalian DNA glycosylases is not exclusive, and further investigations of putative 5mC glycosylase co-factors and/or post-translational modifications are required to define these mechanisms [145]. The glycosylases SMUG1, UNG2, NEIL1 and NTHL1 have also been implicated in the processing of mismatched hmU:G and T and in the processing of intermediate substrates such as 5-carboxyU (Smug1) and 5-formyl-U (Nthl1) [148, 150] (Table 2).

Table 2. Human DNA glycosylases and their known substrates and functions

Gene symbol	Gene name	Expression	DNA Substrates	Mouse Knock-out phenotype
TDG	Thymine DNA glycosylase	Nucleus	T:G [151] 5hmU:G [152] dsDNA	Embryonic lethal between E10.5-11.5, abnormal DNA methylation and impaired heart, vascular and limb development [146, 153].
MBD4	Methyl-CpG binding domain protein 4	Nucleus	TpG [151] 5hmU [154] 5fU [154] dsDNA	Viable and fertile. Increased rate of C to T mutation at CpG dinucleotides [104, 105].
SMUG1	Single-strand-specific monofunctional uracil-DNA glycosylase 1	Nucleus	5hmU [154, 155] 5fU [154] 5caU [156] ss and dsDNA	Ablation of base-excision repair in hmU excision and reduced cellular sensitivity to 5-hydroxymethyluridine toxicity [157].
UNG	Uracil DNA glycosylase	Nucleus (UNG2) Mitochondria (UNG1)	5hmU [132] ss and dsDNA	Increased post-ischemic brain injury [158]. Elevated level of uracil into DNA of dividing cells; mutations at C/G pairs are shifted towards transitions in hypermutation of immunoglobulin genes; reduced class-switch recombination [159].
NEIL1	Endonuclease VIII-like	Nucleus, cyto-	5hmU [132]	Severe obesity, dyslipidemia and fatty liver disease; tend to develop hyperinsulinemia;

	glycosylase 1	plasm Mitochondria	ss and dsDNA	elevated mtDNA damage and deletions; sporadic symptoms of decreased subcutaneous fat, skin ulcers, joint inflammation, infertility and tumours; obesity in male heterozygotes [160].
NTHL1	Endonuclease III-like 1	Nucleus and Mitochondria	T [151] 5fU [154] 5hmU [132] dsDNA	Viable and fertile; slower hepatic repair of thymine glycol DNA lesions under X-ray irradiation [161].

There are other five human DNA glycosylases OGG1, MYH, MPG, NEIL2 and NEIL3 not included in this table as they have not been reported to excise DNA substrates that are involved in model pathways of active DNA demethylation to date.

Functional Interaction of DNA Methylation Mediators in DNA Damage Response and Regulation of DNA Methylation Activity

Aberrant expression of oncogenes and/or silencing of tumour suppressors require additional factors to synergistically break DNA damage response (DDR) barriers for tumour initiation and progression [162]. Functional interaction of DNA methylation mediators may have important roles in these processes. For example, *DNMT1* is a positive transcriptional target of BRCA1, and its decreased expression was shown in *Brca1*$^{\Delta 11}$/$^{\Delta 11}$;*p53*$^{+/-}$ mouse mammary glands and in several human clinical samples [163]. These data support an association between the direct role of DNMT1 methyltransferase activity and global DNA hypomethylation, genomic imprinting loss and an open chromatin configuration [163]. In addition to this direct impact via DNMT1 methyltransferase activity, DNMT1 is associated with the p53 apoptosis pathway via interactions with MBD4 and its protein partner MLH1 in *Xenopus* embryos and mammalian cells [164-166]. DNMT1 forms a trimeric complex with UHRF1 and the deubiquitinating enzyme USP7 on chromatin during cell proliferation [167, 168]. The methyl-CpG binding glycosylase MBD4 interacts with and recruits USP7 to heterochromatic foci, where it physically associates with UHRF1 and DNMT1, indicating that MBD4 regulates DNMT1 activity [169]. These data indicated a prospective functional link between interactions of DNA methylation mediators and BRCA1-associated genome instability via the p53 DDR pathway, alluding to possible epigenetic roles in transformation and aggression of BRCA1-deficient cancers.

Activation of the DDR and apoptosis, G2/M arrest and enhanced radiosensitivity (ATM-p53 apoptosis) were also demonstrated in cancer cells with depleted *UHRF1* [170-172]. In these experiments, UHRF1 and USP7 participate in M phase-specific signalling during cell proliferation, which regulates UHRF1 stability according to the deubiquitinase activity of USP7 and the counteracting site-specific phosphorylation of UHRF1 within its SRA domain [168]. Previous studies showed that the SRA domain of UHRF1 is responsible for binding to methylated CpG [111], and its preferential affinity is dependent on the presence of hemimethylated DNA. Accordingly, UHRF1 and DNMT1 complexes co-localise to replicating heterochromatin and play essential *in vivo* roles in maintaining global and local DNA methylation. Genetic studies showed that *Np95* (*UHRF1*) depletion resultes in a lethal phenotype during early gestation, which resembles multiple defects observed in *Dnmt1*$^{-/-}$ embryos, with growth retardation and excess apoptosis [78, 111]. Further experiments are required to examine the roles of these functional protein complexes in the propagation and preservation of epigenetic signatures and in cellular surveillance systems that respond to intrinsic and extrinsic DDR signals. Because defects or imbalances of epigenetic systems are known to undermine cell viability [40, 173], consequent epigenome instability and impaired DDR in BRCA1-deficient cells with genetic instability may contribute to disease susceptibility, loss of heterozygosity and/or increased phenotypic variation in certain subsets of human breast cancers

DNA Methylation and Cancer Genome Instability

DNA Methylation and Chromatin Structure

A more general role of DNA methylation in genome stability may achieve through chromatin structure modelling, which is the major effect of methyl groups [174]. Although the precise mechanisms by which DNA methylation affects chromatin structure remain elusive, it is accepted that sequence-independent methyl moieties have a direct role in generating a closed chromatin structure [175, 176]. DNA methylation may shape chromatin and gene expression states through an intrinsic effect on nucleosome structure and/or by regulating other factors that displace nucleosomes [177-179]. Additionally, MeCPs specifically recognise DNA methylation and these factors may recruit histone modifiers or chromatin remodellers to shape local chromatin structure. DNA methylation inhibits the binding of chromatin protein CTCF [180]. Moreover, recent studies showed that a group of unmethylated CpG binding proteins that carry CXXC domains, including CFP1, interpret recruitment signals of hypomethylated CGIs and act as transcriptional activators [181, 182]. In principle, an open chromatin structure that determines active gene expression states may increase DNA accessibility to damage and potentially destabi-

lise enzymatic transactions. Therefore, the major role of DNA methylation in shaping chromatin structure places its association with genome integrity in perspective. It has been estimated that hydrolytic deamination of cytosine in single-stranded regions of DNA occurs at least 100-fold more rapidly than in double-stranded DNA [183, 184], indicating that chromatin structure configuration at replication forks or transcription bubbles may determine local DNA vulnerability to damage factors. Moreover, recent studies have discovered and established the significance of many destabilising enzymatic transactions at replication forks following replication stress [185-187]. Some epigenetic factors including demethylase complexes have been implicated at these open chromatic sites [186, 187], but their functional roles in coordinating DNA replication and transcription for genome stability have not been resolved. A better understanding of the significance of DNA methylation machinery and chromatin structure in maintaining genome integrity will facilitate future investigations to target DNA methylation and its mediators for novel drugs and chemotherapeutic combinations.

Promoter Hypermethylation of Tumour Suppressors

It is widely accepted that promoter hypermethylation of key tumour suppressor genes is a driving phenomenon in tumourigenesis [188] and that several DNA repair and cell cycle regulatory genes are common targets of promoter methylation in cancers and are extensively associated with genomic instability [67]. For example, a single-nucleotide variant in the promoter of the mismatch repair gene *MLH1* reduces its activity in transfection reporter assays [189]. Moreover, the comparatively lower activity of the *MLH1* allele was correlated with hypermethylation of its promoter, and transcriptional silencing was demonstrated in somatic cells of some colorectal cancer patients [189]. Epigenetic inactivation of *MLH1* causes the microsatellite instability phenotype in association with colorectal, endometrial and other cancers, resulting in downstream genetic mutations that contribute to genome-wide instability [67]. Therefore, *MLH1* silencing by promoter hypermethylation may be an early carcinogenic event and represents an epigenetic character of colorectal cancer patients with the CpG island methylator phenotype [190]. This epigenetic mechanism was demonstrated in the human colon cancer cell line HCT116, in which epigenetic silencing of *MLH1* by promoter hypermethylation in one allele and genetic mutation in the other results in complete inactivation of *MLH1* [191]. Although enhanced methylation (3%) occurs during *in vitro* passaging of culture cells [192], *de novo MLH1* promoter methylation in cell lines is representative of that in primary tumour cells [193]. Similarly, *CDKN2A*, which encodes the cyclin-dependent kinase inhibitor p16, bears genetic mutations in one allele in HCT116 cells, and promoter hypermethylation of the other allele leads to complete inactivation of p16 [191] and misregulation of the cell cycle [67]. Moreover, MeCPs bind to methylated promoters of *MLH1*, *p16(INK4a)* and master cell cycle regulator and tumour suppressor *BRCA1* and recruit repressive complexes that specifically repress target genes [194, 195]. Accordingly, promoter hypermethylation and silencing of *MLH1* and *CDKN2A* and the key tumour suppressors *BRCA1*, *p53* and *RB1* represent a paradigmatic association between DNA methylation and cancer genome instability.

Gene Body Methylation

Gene body methylation, which occurs mostly in CpG-poor gene exons, is responsible for frequent C to T transition mutations in germ-line and somatic cells of many cancers [193]. Unlike DNA promoter hypermethylation, gene body methylation is not associated with transcriptional repression, but causes gene activation [196], as confirmed in a human active X-chromosome model [197]. Methylation of gene body and orphan CGI promoters benefits transcriptional elongation by suppressing alternative promoter activation [14]. However, CpG methylation blocks elongation in the lower organism *Neurospora crassa*, suggesting that the positive correlation between transcription elongation and CGI methylation in gene bodies is limited in higher organisms such as mammals [193]. In contrast, gene body methylation outside CGIs is considered a major mechanism for silencing repetitive DNA elements such as transposons [198]. Recently, control of alternative splicing by gene body methylation was proposed and is supported by whole-genome studies that show greater methylation in exons than introns and exon–intron boundaries with distinguishing methylation levels [41, 199]. Taken with whole exome sequencing studies that identified numerous gene body mutations of genomic and epigenomic significance, these studies suggested that gene body methylation plays more prominent roles than previously thought [67] Additionally, the presence of 5hmC in gene bodies was consistently associated with gene expression in a number of studies [200-203], further supporting the hypothesis that 5hmC functions as a separate epigenetic mark.

5mC as Mutagen and Cancer-Causing Mutation

5mC is prone to spontaneous deamination and is a prominent source of germ-line and somatic muta-

tions [204, 205]. Specifically, mutation of 5mC to T occurs with 10–50-fold greater frequency than other transitions [206], and mutation rates at CpG sites are estimated to be at least 10–18-fold more frequent than at non-CpG dinucleotides [207-211]. Moreover, DNA bases in CpG dinucleotides are vulnerable to chemical reactions, and endogenous and exogenous stress may favour spontaneous deamination via direct or indirect influence. The extracyclic amino group at C4 position of cytosine is subject to hydrolysis that is sensitive to salts, ATP and pH under physiological conditions [212]. Additionally hydrolysis efficiency can also be changed by exposure to exogenous stimuli such as xenobiotics or reagents generating reactive oxygen species [213]. Hydrolytic deamination of cytosine and 5mC results in pairing of uracil (U) and T with guanine (G), leading to the mismatches G:U and G:T, respectively. A failure to repair these mismatches prior to DNA replication causes cell acquiescence and mutation from C:G to T:A. Mutations at methylated CpG sites reportedly contribute one-third of all pathophysiological mutations and include familial mutations and single nucleotide polymorphisms (SNPs) in somatic cells [67]. A number of mutation hotspots are frequently found in DNA methylation mediators such as *DNMT3A* in acute myeloid leukaemia (AML) [82], *DNMT1* in colorectal cancer [69] and *DNMT3B* in immunodeficiency-centromeric instability-facial anomaly (ICF) and chromosome instability syndromes [87]. Moreover, C to T transitions at methylated CpG sites increase numbers of natural p53 point mutations by as much as 50% in colorectal cancers and significantly increase the incidence of predominant *p53* mutations in breast and ovarian cancers [205, 214]. In addition, promoter methylation of *BRCA1* and hotspot mutations in the *BRCA1* gene body (due to methylation of exons and subsequent deamination of 5mC) are frequent in breast and ovarian cancers [215], indicating another association between DNA methylation, the p53 DDR pathway and BRCA1-associated breast cancers.

Final Remark

Genome integrity is an absolute requirement of intact systems in higher organisms. Various forms of DNA methylation and respective mediator proteins may act as a multi-reacting processor and reflector of other epigenetic events such as histone modification and chromatin remodelling. These DNA modifications respond to extra-nuclear signals via sensor proteins and control gene expression and chromatin changes accordingly, providing feedback mechanisms that recruit effectors of transcription and DNA repair and replication and that play vital roles in both development and disease. Functional characterisation of the associated proteins continues to be an area of high interest, and accumulating *in vivo* data contribute an increasingly precise understanding of DNA methylation modes. These insights linking the genetic instability and epigenetic perturbations such as aberrant DNA methylation machinery may ultimately form the basis for novel therapeutic strategies and targets for the treatment of inherited, acquired and malignant diseases.

Abbreviations

5mC: 5-methylcytosine; DNMTs: DNA methyltransferases; MeCPs: methyl-CpG binding proteins; BER: base excision repair; TETs: ten-eleven translocation cytosine dioxygenases; TSSs: transcriptional start sites; CGIs: CpG islands; 5hmC: 5-hydroxymethylcytosine; 5fC: 5-formylcytosine; 5caC: 5-carboxylcytosine; TDG: thymine DNA glycosylase; ES cell: embryonic stem cells; DNMT1: DNA methyltransferase-1; MBD proteins: methyl-CpG binding domain proteins; 5meCpG: 5-methyl-CpG; SRA domain: SET and RING finger-associated domain; PHD domain: plant homeo domain; TRD domain: trans-repressor domain; DSBH: double-stranded β–helix; 5hmU: 5-hydroxymethyluracil; CXXC: cysteine–X–X–cysteine; αKG: α-ketoglutarate; AP: abasic apurinic/apyrimidinic; DME: DEMETER; SNP: single nucleotide polymorphism; AML: acute myeloid leukaemia; ICF: immunodeficiency-centromeric instability-facial anomaly; DDR: DNA damage response; BRCA1: breast cancer1, early onset.

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Competing Interests

The authors have declared that no competing interest exists.

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