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Making it or breaking it: DNA methylation and genome integrity

Anusha Sriraman[1,2], **Turja K. Debnath**[1,2], **Blerta Xhemalce**[1,2,3], **Kyle M. Miller**[1,2,3]

[1]Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX 78712, U.S.A.;

[2]Institute for Cellular and Molecular Biology, The University of Texas at Austin, Austin, TX 78712, U.S.A.;

[3]Livestrong Cancer Institutes, Dell Medical School, The University of Texas at Austin, Austin, TX 78712, U.S.A.

Abstract

Cells encounter a multitude of external and internal stress-causing agents that can ultimately lead to DNA damage, mutations and disease. A cascade of signaling events counters these challenges to DNA, which is termed as the DNA damage response (DDR). The DDR preserves genome integrity by engaging appropriate repair pathways, while also coordinating cell cycle and/or apoptotic responses. Although many of the protein components in the DDR are identified, how chemical modifications to DNA impact the DDR is poorly understood. This review focuses on our current understanding of DNA methylation in maintaining genome integrity in mammalian cells. DNA methylation is a reversible epigenetic mark, which has been implicated in DNA damage signaling, repair and replication. Sites of DNA methylation can trigger mutations, which are drivers of human diseases including cancer. Indeed, alterations in DNA methylation are associated with increased susceptibility to tumorigenesis but whether this occurs through effects on the DDR, transcriptional responses or both is not entirely clear. Here, we also highlight epigenetic drugs currently in use as therapeutics that target DNA methylation pathways and discuss their effects in the context of the DDR. Finally, we pose unanswered questions regarding the interplay between DNA methylation, transcription and the DDR, positing the potential coordinated efforts of these pathways in genome integrity. While the impact of DNA methylation on gene regulation is widely understood, how this modification contributes to genome instability and mutations, either directly or indirectly, and the potential therapeutic opportunities in targeting DNA methylation pathways in cancer remain active areas of investigation.

Introduction

Genetic information is stored in DNA, which must be protected from mutations and alterations that can disrupt cell homeostasis and promote diseases. The integrity of the

Correspondence: Kyle M. Miller (kyle.miller@austin.utexas.edu).

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genome is constantly exposed to various threats including genotoxic agents that can harm the stability of the genome. A few examples include exposure to ultraviolet radiation (UV) from the sun, ionizing radiation (IR), natural products or manmade drugs used during cancer treatments and intrinsic cellular processes that damage DNA including replication errors, metabolic products and alterations in proteome homeostasis [1–3]. Exposure to these endogenous and exogenous DNA damaging agents can result in mutations leading to DNA base changes (i.e. via deamination); impact replication through formation of non-canonical DNA structures including RNA-DNA hybrids (R-loops) and G-quadruplexes; modulate gene expression through changes in methylation patterns at the promoter or gene body and form dangerous DNA lesions such as DNA double-strand breaks (DSBs), all of which can threaten genome integrity (Figure 1) [2,4,5]. To combat these hazards, cells utilize diverse mechanisms that are collectively termed as DNA damage responses (DDR), which act to sense DNA damage and repair it, while coordinating these activities with cellular processes including cell cycle, replication/transcription, programmed cell death or senescence. The essential nature of these balanced pathways in genome integrity are highlighted by the frequent loss of these processes in cancer, in which genome instability is a hallmark observed broadly across many cancer types [6].

DNA methylation is a dynamic epigenetic mark mediated by DNA methyltransferases (DNMTs). While promoter methylation is generally associated with transcriptional gene silencing, gene body hypermethylation correlates with gene activation [7]. Methylation is also essential for X-chromosome inactivation, development and differentiation [8]. Since DNA methylation modifies the potential function and physical properties of the base, changes in methylation could also influence genome integrity and cancer by altering various processes either directly through mutations involving base changes and coding outcomes or more broadly through the DDR and DNA repair.

DNA methylation

Methyltransferases

DNA methylation is catalyzed by a family of DNMTs. DNMTs mediate the transfer of the labile methyl group (-CH_3) from S-adenosyl-L-methionine (SAM) cofactor to the C5 position of the cytosine ring forming 5-methylcytosine (5mC) (Figure 2A). Mammalian DNMTs include DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L. DNMT1 is a large protein that preferentially binds to hemi-methylated CpG dyads through its functional partner Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1). This complex functions to maintain DNA methylation during DNA replication [9,10]. *De novo* DNA methylation activity is catalyzed by DNMT3A and DNMT3B, which are responsible for establishing methylation patterns during development [11]. The other member of the DNMT3 family is DNMT3L, which is catalytically inactive and is required for gene imprinting, and regulation of DNMT3A/B activity [12,13]. Interestingly, DNMT2 has no DNMT activity, although it shares sequence similarities with other DNMT family members, and was subsequently identified as an RNA methyltransferase. DNMT2 has been shown to catalyze the methylation of $tRNA^{Asp}_{GUC}$ at position 38 [14] and other tRNAs (reviewed in [15]) to yield 5-methylcytosine (m^5C).

DNA demethylation

The mechanism of DNA demethylation remained a puzzle until the characterization of Ten-Eleven Translocation proteins (TET) in 2009 [16,17]. TET proteins (TET1, TET2 and TET3) are Fe(II) and α-ketoglutarate-dependent dioxygenases. They utilize molecular oxygen and α-ketoglutarate as co-substrates to generate oxidized 5mC derivatives along with succinate and carbon dioxide (CO_2) as co-products. Using recombinant TET proteins, it was confirmed that they not only oxidize 5mC to 5-hydroxymethylcytosine (5hmC), but also mediate the subsequent oxidation of 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) [18,19] (Figure 2A). 5fC and 5caC are recognized and excised by thymine DNA glycosylase (TDG), which generates apyrimidinic (AP) sites [20] that are corrected by Base Excision Repair (BER). Recently, it was shown that GADD45A and GADD45B form a complex with TDG and TET2, which promotes DNA demethylation *in vitro* [21]. Activity-induced cytosine deaminases (AIDs) and apolipoprotein B mRNA editing complex (APOBEC) mediates an alternate path to successive oxidation of 5mC and 5hmC [22]. In this pathway, the modified nucleotide is also repaired by BER. The above processes are termed as active DNA demethylation, since it involves the removal of a methyl group by enzyme-initiated reactions. Passive DNA demethylation can also occur when the methyl group of 5mC is lost due to inhibition of DNMT1 followed by successive rounds of DNA replication, which dilute out the methylated DNA [23,24].

DNA methylation readers

Methylated DNA is recognized by methyl-CpG-binding domain (MBD) proteins, UHRF proteins, zinc-finger-containing proteins, and the more recently identified basic leucine-zipper (bZIP) and homeodomain-containing transcription factors (TFs) (Figure 3) [25–28]. MBD-containing proteins encompass MeCP2, MBD1, MBD2, MBD3 and MBD4. The MBD domain of MeCP2 is highly specific to methylated CpG sites, which are adjacent to an A/T-rich sequence [29]. MBD1 has been shown to interact with MBD1-containing chromatin-associated factor (MCAF-1) and histone methyltransferases like Suv39H and SETDB1. Furthermore, MBD1 has been classified as an oncogene or tumor suppressor depending on tumor type [30]. MBD2 is a subunit of the Mi2-NuRD complex that facilitates repression of genes upon its recruitment to methylated promoters [31]. Moreover, MBD2 has been shown to play a key role in the maintenance and spread of DNA methylation [32]. MBD3 and MBD4 are unusual regarding their DNA binding activities, as MBD3 cannot bind DNA directly due to a mutation in the MBD domain and is often found working in concert with MBD2 to enhance its recognition [33,34], while MBD4 binds to DNA but preferentially recognizes a guanine mismatched with thymine, uracil or 5-fluorouracil [35]. MeCP2 is also involved in the recruitment of DNMT1 to hemi-methylated DNA [36].

UHRF consists of two proteins that include UHRF1 and UHRF2, which bind 5mC and 5hmC via a SET- and RING-associated DNA-binding domain, respectively [10,37,38]. UHRF proteins aid in maintaining DNA methylation by targeting DNMT1 specifically to hemi-methylated DNA during DNA replication [10]. The zinc-finger domain proteins composed of ZBTB33 (Kaiso), ZBTB4 and ZBTB38 bind to methylated DNA and repress transcription in a DNA methylation-dependent manner [39–41]. Other members of this family include ZFP57, KLF4, WT1, EGR1 and CTCF [25,30]. Aside from ZFP57, all these

proteins have shown marginal selectivity toward mCpG (~1.5–1.8 fold) over CpG *in vitro*, and in cells [25]. In the case of CTCF, binding to methylated DNA has been shown to depend on the position of the CTCF motif recognition [42]. Cytosine methylation status at position 12 of the core consensus motif increased CTCF binding, but was severely reduced when position 2 was methylated [43,44]. Furthermore, CTCF has been shown to maintain genome stability through DSB repair by homologous recombination (HR) [45,46]. These studies revealed that the DNA binding domain of CTCF was required for interacting with DNA lesions. This suggests the potential for DNA methylation to affect CTCF binding and its associated repair process. For example, DSBs are repaired by two main pathways, non-homologous end joining (NHEJ) and HR. Given that CTCF binding is reduced by DNA methylation and CTCF promotes HR specifically, this suggests that breaks within hypo-methylated DNA would be preferentially repaired by HR. DNA methylation changes occurring *de novo* at break sites could also impact the dynamics of repair. CTCF may also influence the three-dimensional organization of the genome that is also known to be involved in genome maintenance [45]. Structural analysis has indicated that transcription factors containing bZIP and homeodomain bind methylated CpG sequences *in vitro* [27,28]. However, *in vivo* characterization of these transcription factors with DNA methylation is required and their potential involvement in genome maintenance remains untested.

DNA methylation of adenine

Although cytosine methylation has been extensively studied, adenine methylation in eukaryotes has only recently been identified using deep sequencing technologies. Adenines in the mammalian genome can undergo a similar SAM-dependent methylation on the exocyclic amino group ($-NH_2$) at the sixth position of the purine ring to form N^6-methyldeoxyadenosine (6mA or m^6dA) that is mediated by a new family of DNMTs (Figure 2B). A recent study using overexpression and silencing experiments reported that N^6-adenine-specific DNMT 1 (N6AMT1) is able to catalyze DNA m^6dA methylation (calculated to be ~0.051% of all adenines in the human genome) [47]. Demethylation of this mark is mediated by an alkylated DNA repair protein B (ALKB) homolog, ALKBH1 in humans [47] (Figure 2B). This study also indicated that decreased levels of genomic m^6dA levels correlated with tumorigenesis and indicated poor prognosis for cancer patients. Other studies have reported a role of human MettL3–MettL14 (which is more commonly known for m^6A deposition in RNA), in adenine methylation on ssDNA, and mtDNA [48,49] (Figure 2B). In mitochondria, hypoxia increased m^6dA levels, suggesting a putative connection between adenine methylation and mitochondrial DNA damage [49]. Besides m^6dA, human N^6-hydroxymethyladenine (hm^6dA) has also been described in human genomic DNA [50]. Oxidation of m^6dA to hm^6dA is catalyzed by human ALKBH1 and its levels increased in lung carcinoma tissues. Thus, the observation of methylated adenines in the human genome, although infrequent, warrants further consideration for its potential involvement in biological processes including the DDR and human diseases such as cancer.

DNA methylation and mutations

In the human genome, 5mC is frequently found in CpG dinucleotides, with these sites being hotspots for mutations including in tumor suppressor genes. For example, CpG island (CGI)

mutations within the coding region of p53, a gene involved in genome stabilization and cell cycle/apoptotic responses, contribute to ~25% of its inactivating mutations in cancer [51]. Mutations within CpG sites can occur as a consequence of exposure to agents that are alkylating, oxidizing and hydrolytic. Hydrolytic deamination of cytosine results in the formation of uracil in DNA, which is readily recognized and repaired by uracil DNA glycosylase (UDG) (Figure 4A). However, in the case of 5mC, deamination forms thymine, a naturally occurring DNA base that makes it significantly more difficult to repair by TDG. Deamination of 5mC results in increased C→T transitions, which are among the largest class of mutations found in human cancers (Figure 4B) [52]. Transition mutations also disrupt DNA methylation patterns, potentially causing aberrant transcription. Both C→T transitions and alterations in DNA methylation can contribute to carcinogenesis. Moreover, the presence of 5mC in mammalian DNA enhances the formation of pyrimidine dimers (CC→TT transitions) upon exposure to UV light from sun, which promotes skin cancer [53]. This is due to the higher energy absorption of 5mC compared with cytosine.

Transposable elements (TEs) present in mammalian genomes also rely on DNA methylation for silencing and repressing transposition. Oncogenesis is characterized by global hypomethylation, which promotes TE reactivation resulting in increased DNA breaks, insertional mutagenesis and genome instability [54] (Figure 4C). Nearly half of all human cancers have been found to express long interspersed element-1 (LINE-1), which are associated with p53 deficiency [55]. Thus, these studies highlight the diverse ways that 5mC can contribute to various types of mutations and alterations in the genome with the potential to cause genome instability and cancer.

DNA replication and DNA methylation

Maintaining and faithfully copying genetic information are an essential requirement for life. During DNA replication, DNMT1 localizes to the replication fork via its interaction with Proliferating Cell Nuclear Antigen (PCNA), the replisome clamp [56]. This interaction allows the maintenance of parental methylation on to newly synthesized daughter DNA strands during replication [9]. Although our cells have developed sophisticated mechanisms to replicate DNA with accuracy, replication is still subject to errors and interruptions. When cells are damaged during S-phase, it often gives rise to intermediates that causes the polymerases at the fork to temporarily cease their activity, known as ‘fork stalling’. Repair mechanisms are usually initiated to allow the fork to continue, but this event can also result in ‘fork collapse’, which ultimately leads to the formation of DSBs that trigger the DDR [57]. ‘Fork stalling’ can occur when the replication fork encounters transcription-replication conflicts such as R-loops, or non-canonical DNA structures like G-quadruples, Z-DNA etc [58]. Engagement of DDR and repair pathways help to alleviate replication stress in cells. There seems to be an intimate link between cell cycle regulation, DDR and DNA methylation. It is conceivable that upon activation of the DDR, the cell cycle regulator p21 or CDKN1A is activated by p53, which disrupts the interaction between DNMT1 and PCNA, suggesting a negative role for p21 in regulating DNA methylation [59]. In addition, the retinoblastoma gene product Rb can also bind to DNMT1 and inhibit its DNMT activities during DNA replication [60]. Moreover, these pathways are frequently dysregulated in cancer, which in turn could impact DNA methylation. The pathways

controlling these proteins are relieved when the damage has been repaired, and this temporary stalling of DNMT1-coupled PCNA might alter DNA methylation maintenance. Thus, it is not well understood whether epigenetic patterns are faithfully maintained after DDR initiation during DNA replication.

Apart from the methylation-dependent activities of DNMT1, a recent study identified the PCNA-binding domain (PBD) of DNMT1 to be associated with increased endogenous DNA damage and mono-ubiquitination of PCNA, a known marker of replication stress, in a methylation-independent manner [61]. This observation suggests the potential for a non-canonical role for DNMT1 that is not related to DNA methylation *per se* in cancer initiation and progression when DNMT1 is overexpressed. While DNA methylation involving DNMT1 is targeted in cancer, the PBD of DNMT1 may provide an additional therapeutic option, which will require additional information on how DNMT1 and its mis-expression can impact genome stability. Another domain of DNMT1, namely the Replication Foci Targeting Sequence (RFTS) has recently been identified to be crucial for maintaining global DNA methylation and genome stability [62]. Here, a direct interaction between the histone marks H3K9me3 and H3 ubiquitylation with the RFTS domain was established through structural, biochemical and cellular analyses. Mutations in this domain led to decreased CpG methylation and increased sensitivity to IR. Therefore, studies directed toward understanding the functionality of the various domains of DNMT1 will be helpful in fully appreciating its multifunctional roles in maintaining genome stability.

DNA methylation and DNA damage responses

There is strong evidence that DNA methylation is associated with the DDR (Figure 5). These observations include; (1) aberrant DNA methylation patterns cause genome instability like mitotic catastrophe in cell lines [63,64]; (2) mice lacking Dnmt1 are genetically unstable [65]; (3) DNMT1 has been shown to be recruited to sites of DNA damage [66–70] and (4) interaction of DNMTs with PCNA aids DNA replication and repair [59,66,67,70].

In more detail, the essential role of DNA methylation was contributed to p53-mediated apoptotic responses in DNMT1-deficient mouse embryonic fibroblasts [71]. Furthermore, inactivation of p53 in DNMT1 KO mice was able to rescue this lethal phenotype. This suggests that loss of DNA methylation leads to activation of p53 responses and/or the DDR. A study on the role of DNMT1 in pancreatic organogenesis observed increased p53 activation on a transcriptional level upon loss of DNMT1 [72]. On the other hand, deletion of DNMT1 in hTERT-immortalized normal human fibroblasts showed hallmarks of mismatch repair (MMR) deficiency [73]. In this case, loss of DNMT1 led to increased resistance to the nucleoside analog 6-thioguanine (6TG), and enhanced mutation rates at microsatellites. Additionally, these cells indicated loss of MMR proteins such as MLH1, PMS2 and MBD4, with increased levels of phosphorylated H2AX (γH2AX), a marker of DNA damage. Interestingly, inhibition of PARP was able to relieve the effects of DNMT1 deletion [73]. Therefore, these studies indicate the significance of DNMT1 in maintaining genomic stability, including through promoting DNA repair.

Another study on DSB repair revealed that upon oxidative stress, the chromatin remodeling complex NuRD mediates transcriptional repression by engaging with DNMT1, DNMT3A and DNMT3B causing abnormal *de novo* methylation [74]. This resulted in silencing of tumor-suppressive genes leading to increased proliferation, invasion and metastasis of colorectal cancer cells upon intrasplenic injection into immune-compromised mice. DNMT1 is also recruited to sites of DNA damage via its PBD, independent of its catalytic activity [66]. In addition to PCNA, DNMT1 interacts with other components of the DNA damage machinery including CHK1 and the 9-1-1 complex [66]. After recruitment to DNA lesions, DNMT1 modulates Ataxia telangiectasia and Rad3-related (ATR) signaling and suppresses abnormal activation of the DDR machinery. DNMT1 is required to repair DSBs as its loss results in delayed kinetics of IR-induced DSBs [63]. However, DNMT1 is only transiently recruited to damage sites, raising the question of whether or not methylation of DNA is its only function, especially during early stages of the DDR. Since PCNA is bound to DNMT1, it is tempting to speculate that DNMT1 restores epigenetic information on the newly synthesized DNA strand at repaired DNA lesion sites.

An additional important question to consider is whether DDR activation and processing of DSBs alters DNA methylation patterns. A study conducted by Cuozzo et al. showed that upon introduction of a DSB using the endonuclease I-Sce1 and gene conversion by HR, there was a concomitant change in methylation patterns pre- and post-DSB repair [75]. They propose that DNMT1 could act as a *de novo* methyltransferase that is recruited to DNA breaks by PCNA to methylate one of the newly synthesized strands causing differential methylation patterns, resulting in a hemi-methylated DNA segment. Upon replication of this region, cells containing both hypomethylated and methylated regions would occur, resulting in differential expression of the GFP reporter. These data suggest that DNA damage could cause alterations in DNA methylation and transcriptional status around the break site. Using a similar system with I-Sce1 and GFP+ selection of HR-repaired breaks, both ‘high’ and ‘low’ GFP expressing cells were isolated and analyzed for *de novo* methylation [70]. Interestingly, DNMT1, UHRF and DNMT3A were found to be recruited to DSB sites and required for methylating DNA at I-Sce1 induced DNA breaks, with DNA methylation patterns being regulated by transcription. Thus, there is strong evidence linking DNA methylation and DNA repair processes, although the molecular function of DNA methylation in DNA repair remains poorly understood. With this picture remaining unclear, further investigations are warranted. Given the prevalence of DNA repair deficiency, genome instability and alterations in DNA methylation in cancer, understanding these links may aid in dissecting their contribution to tumor development and their potential involvement in anti-cancer therapies.

TET enzymes have been shown to be critical for promoting genome integrity during replication stress [76]. Depletion of TET in mouse embryonic stem cells (mESCs) led to mitotic abnormalities upon aphidicolin treatment. Furthermore, 5hmC induction at sites of aphidicolin- and laser micro-irradiation-induced DNA damage occurred in a TET-dependent manner. Another report identified TET3 as an ATR target leading to DNA demethylation with increased 5hmC levels upon UV and camptothecin (CPT) exposure [77]. Depletion of TET3 resulted in defects in repair of UV and CPT lesions as well as survival from these DNA damaging agents. These results suggest DNA demethylation by TET3 as a requisite

step in repairing these types of DNA lesions. TET1 has been found to be a target of Ataxia Telangiectasia Mutated (ATM). While irradiation increased 5hmC in neurons and fibroblasts, this response was lost in ATM-deficient cells [78]. It has been proposed that 5hmC loss due to ATM deficiency may preferentially affect cerebellar Purkinje cells, linking defects in TET1 and 5hmC to ataxia-telangiectasia disorder that results from ATM defects and resulting in neurodegeneration. Thus, TET proteins and 5hmC play an important role in regulating the DDR through DNA damage signaling and DNA repair.

DNA methylation and carcinogenesis

DNA repair machinery has evolved to maintain genomic integrity by suppressing the formation of mutations. Epigenetic silencing of DNA repair proteins can result in cells deficient for these repair pathways, resulting in mutations that promote carcinogenesis [79]. Aberrant methylation at the promoter CGIs within the promoter of key genes can lead to alterations in gene expression and defects in cellular pathways. Similarly, mutations in driver genes can result in downstream changes in DNA methylation that contribute toward oncogenesis. For example, mutations in the gene Isocitrate dehydrogenase 1 (*IDH1*) in glioblastoma patients result in abnormal production of 2-hydroxyglutarate. This leads to a CGI methylator phenotype (CIMP) that remodels the methylome and transcriptome due to inactivation of TET-mediated demethylation pathway [80]. The aberrant regulation by DNA methylation on the p53 gene remains controversial due to a lack of direct methylation over the p53 core promoter. Multiple investigations have been conducted to identify relationships between the mutation status of p53 and tumor grade with promoter DNA methylation in cancers [81,82]. However, no clear correlations were recognized, indicating that the primary mechanism of transcriptional silencing of the p53 promoter does not seem to depend on DNA methylation. In the case of BRCA1, methylation of CpG sites close to the transcriptional start site (TSS) is associated with reduced mRNA and protein levels [83]. Additionally, functional loss of BRCA1 involves methylation of a single copy of BRCA1, followed by loss of heterozygosity (LOH) event. This results in loss of HR activity with a pattern of genome-wide mutations and genome instability [84].

Aside from gene silencing by methylation, mutations or loss of methylation writers or erasers can also contribute to mutagenesis. Defects in DNMT1 have been reported to have a significant impact on microsatellite instability (MSI), a hallmark of MMR deficiency [85]. Deficiency of DNMT1 triggers defects in MMR through reduced levels of repair proteins like MLH1, PMS2 and MBD4 [73]. Somatic missense mutations in DNMT3A have been reported in ~20% of Acute Myeloid Leukemia (AML) patients and mutations are also observed in other hematological malignancies [86–89]. These mutations have been associated with poor overall survival in AML patients [90]. Although the downstream effects of DNMT3A mutations in AML are not well understood, a recent report observed association between DNMT3A and the leukemogenic HOX cofactor MEIS1, in the absence of Mixed Lineage Leukemia (MLL) fusions [91,92]. These findings may suggest a connection between altered DNA methylation through DNMT3A mutations and other transcriptional regulators, including MEIS1. Mutations in methylation erasers like TET2 are frequent in a wide spectrum of myeloid malignancies, causing aberrant DNA methylation patterns [93]. These mutations impair the catalytic activity of TET2 *in vitro* and *in vivo* [94].

Since TET2 converts 5mC into 5hmC, inactivating TET2 mutations would contribute to increased 5mC in the genome. Contrasting roles for TET1 have been reported. On one hand, the loss of Tet1 in mice leads to the development of B-cell lymphoma, suggesting a tumor suppressive role [95]. On the other hand however, TET1 can act as an oncogene since it is also a transcriptional target of MLL fusion proteins that activate the expression of downstream oncogenic targets to promote leukemogenesis [96]. Additional work is required to further establish how alterations in DNA methylation and demethylation perturb normal cellular functions, including those involved in the DDR, which could impact tumorigenesis and genome stability. Given that increased DNA damage is prevalent in cancer, it cannot be ruled out that DNA methylation at breaks sites, if impaired, could also directly be involved in cancer promoting events including mutagenesis.

Targeting DNA methylation

Inhibition of DNMTs can lead to increased expression of tumor suppressor genes along with a reduction in tumorigenicity in some settings [97]. These findings have made DNMTs a valuable target for cancer therapy. Indeed, the first Federal Drug Administration (FDA) approved drugs targeting an epigenetic pathway were DNMT inhibitors [98]. Commonly used catalytic inhibitors of DNMTs are nucleoside analogs consisting of 5-azacytidine (azacitidine), 5-aza-2′-deoxycytidine (decitabine) and zebularine. Currently, both azacitidine and decitabine are FDA approved for use against myelodysplastic syndrome (MDS) and decitabine for AML. Azacitidine and decitabine are nucleoside inhibitors that are incorporated into replicating DNA [99]. When DNA is methylated, the presence of azacytidine prevents the resolution of a covalent reaction intermediate, leading to the formation of protein-DNA adducts [100]. This results in decreased DNMT activity while also increasing the presence of protein-DNA adducts, which can lead to DNA damage and impair DNA replication [101,102]. Despite some poor outcomes against tumors upon using catalytic inhibitors of DNMTs, these compounds are able to demethylate and reactivate tumor suppressor gene expression in solid tumors [103]. Zebularine (1-(β-D-ribofuranosyl)-2(^{1}H)-pyrimidinone) is an orally bioavailable DNMT inhibitor [104]. Treatment in cell systems indicates complete demethylation by depleting DNMT1 and partially depleting DNMT3A and DNMT3B [105,106]. These compounds exemplify the active area of research in drug discovery and cancer therapeutics focused on targeting DNA methylation.

Combinations of azacytidine or decitabine with standard chemotherapy have shown increased clinical activity. For example, co-treatment of cisplatin and 5-azacytidine treatments revealed an increase in DNA lesions that triggered the activation of DDR pathways [107]. Cisplatin and decitabine co-treatment resulted in partial response in one patient with cervical cancer and two minor responses - in one patient with non-small cell lung cancer and the other with cervical cancer [108]. However, it has to be noted that this combination led to significant hematological toxicity. Treatment with decitabine rescued cisplatin resistance in head and neck squamous cell carcinoma, leading to reduced tumor growth and reduced dosage of cisplatin in a xenograft model [109]. Further analysis revealed differences in methylation patterns between cisplatin-sensitive and cisplatin-resistant patient tumors, suggesting a role for gene methylation arrangements as possible biomarkers for

cisplatin resistance. Apart from methylation-dependent effects on drug combinations, activation of signaling pathways can result in drug sensitivity. Cytotoxicity mediated by cisplatin or doxorubicin was found to be augmented by decitabine addition in bladder cancer cells by activation of Hippo pathway through RASSF1A [110]. An ongoing clinical trial (NCT03467178) is studying the combination of decitabine and carboplatin in platinum-resistant ovarian cancers [111]. While some promising synergistic tumor suppressive phenotypes have been observed when combining DNA damaging agents and DNA methylation inhibitors, mechanisms explaining these connections remain incomplete.

PARP1, a poly (ADP-ribose) polymerase involved in gene expression and the DDR, and DNMT1 have been found to interact, which may provide a direct link between the DDR and DNA methylation [112]. Combination of low doses of PARP and DNMT1 inhibitors have shown increased retention of PARP1 and DNMT1 at laser-damaged sites with increased binding of PARP1 to chromatin. This combination of inhibitors led to increased frequency of DSBs and synergistic cell death in AML cell lines, primary cells and mouse xenografts. In addition to PARP trapping on to chromatin by PARP inhibitors, PARP1 has also been shown to antagonize DNA end-resection in DSB repair [113] and also promote NHEJ [114]. Together, the function of PARP in the DDR is likely to impact cell death and sensitivity to other inhibitors, including DNMT inhibitors, in several ways that future work is needed to decipher. Interestingly, a recent investigation disclosed treatment with DNMT1 inhibitors led to hypermethylation of certain CGIs corresponding to genes differentially expressed in cancer tissue such as NFAT, LEF1 and MAZ-regulated [115]. This suggests that these inhibitors possess a complex mechanism of action and a deeper understanding of the response to DNMT1 inhibitors at the gene level is necessary to understand both their effects on the DDR and how combination of therapies can result in therapeutic benefit. Apart from the canonical DNA methylation inhibitors, targeting the ability of DNMT1 to interact with PCNA may provide another avenue to inhibit pro-tumorigenic functions of DNMT1 [61]. In this study, DNMT1 overproduction led to increased endogenous DNA damage in a methylation-independent and PBD-dependent manner, which also resulted in increased mutations, a cancer promoting process. The PBD of DNMT1 may represent an actionable drug target that could be pursued in future studies. The development and use of these drugs may also alleviate potential side effects of catalytic DNA methylation inhibitors.

Concluding remarks

Epigenetic patterns like DNA methylation serve as important regulatory roles in DNA-based processes, including transcription, replication and DNA repair. DNA methylation serves as a conduit between gene expression and important regulatory mechanisms that govern genome integrity. Aberrant DNA methylation patterns like global hypomethylation and promoter hypermethylation are observed in tumors and are linked with cancer progression. This makes it unsurprising that their activities are frequently modulated during tumorigenesis, making them potential ‘druggable’ targets. Although DNA methylation and its associated regulatory factors have been widely studied, it remains unclear their precise role in genome integrity pathways, including the potential link between DNA methylation and genome instability in cancer.

Does DNA methylation play a specific role during DNA repair and how does this contribute to epigenetic stability? A study has linked the recruitment of DNMT1 and MMR proteins MSH2 and MSH6 to the chromatin in response to oxidative damage [116]. Interestingly, they observed a reduction in nascent transcription after H_2O_2 treatment, which was abrogated upon knockdown of DNMT1 and/or MSH6. Furthermore, catalytically inactive DNMT1 was also recruited to chromatin, and could interact with MSH2/MSH6 upon oxidative damage. This suggests that the methylation activity of DNMT1 is likely not required, at least at the level of DNA lesion recognition. Additionally, reduction in transcription at sites of damage prevents interference between transcription and repair processes [117,118]. Is it possible that in addition to repair of the break, epigenetic modifications including DNA methylation marks are restored at repaired lesions? It has been noted that breaks occurring at gene promoters are most often repaired with no promoter hypermethylation and removal of silencing factors, including through the actions of the deacetylase SIRT1, but they are occasionally retained, which results in sustained gene silencing [68]. This suggests that repair of DNA breaks may lead to heritable silencing of CGI-containing promoters. Methylation of promoter CGIs is frequently associated with gene silencing and cancer.

Another question is whether DNA methylation in cancer cells directly affects repair processes or indirectly through gene expression alterations, or both. Reported data that could address this point is complex. The deficiency of multiple repair genes is often observed in cancers, including MLH1, O^6-methylguanine-DNA methyltransferase (MGMT) and MSH2 in gastric cancers due to aberrant methylation patterns [119]. This can occur directly by promoter hypermethylation of repair proteins, e.g. MLH1 or other indirect mechanisms including overexpression of miRNAs that control the expression of many cellular proteins, e.g. miR-155 that targets MLH1 gene for repression [120]. Faulty DNA repair leads to increased mutations and epigenetic alterations, central to causing genome instability. Investigations on the role of chromatin regulator ATRX in DNA repair identified the role of its PCNA-interacting protein (PIP) motif for efficient HR-repair in G_2 phase of the cell cycle [121]. This could suggest a plausible role for DNMT1 in mediating HR since it also binds to PCNA and conversely, DNMT1 overexpression may affect DNA synthesis during HR. Another repair pathway mediated by PCNA is MMR, which requires interaction between the PIP-motifs of MSH2 and MSH6 for identification of the mismatch and loading of RFC1 that acts as a processivity factor for DNA polymerases DNA pol δ and pol [122]. Thus, DNMT1 overexpression may impact other PCNA-dependent repair pathways, in addition to replication.

At the genomic level, DNA methylation within gene bodies is positively correlated with expression levels. Although the function of intergenic DNA methylation is not clearly understood, recent studies suggest that a loss of gene body methylation could result in activation of unscheduled intragenic transcription [123], and alternative promoter activation [124]. In addition, a casual relationship between gene body methylation and transcription has been identified. Treatment with azacytidine led to demethylation of gene bodies, resulting in reduced transcription of oncogenes, including c-MYC [125]. Thus, demethylation inhibitors seem to not only cause promoter demethylation, but also gene body

demethylation. Alterations in these pathways could increase transcriptional noise, and possibly interfere with the DDR and genome stability.

Connections between DNA methylation and R-loops, a structure that can affect DNA template processes, have been reported. R-loops are enriched at promoter CGIs, and how these are recognized and modulated by epigenetic readers is not well known. Recently, it was shown that GADD45A could bind directly to R-loops formed by long non-coding RNAs (lncRNAs) and mediate DNA demethylation by recruiting TET1 [126]. This opens other questions such as whether GADD45A specifically recognizes R-loops at lncRNAs and whether other DNA methylation readers exist which can identify R-loops formed due to transcription-replication conflicts. Aside from R-loops, single-stranded guanine-rich DNA sequences can form G-quadruplexes, which are four-stranded DNA structures [127]. They are usually found at active chromatin and were found to sequester DNMT1 leading to a local inhibition of DNMT1 function at CGIs [128]. This suggests that G-quadruplex structures protect certain CGIs by sequestering DNMT1, and may play an important role in establishing the epigenome at these loci, although connections between DNA methylation, G-quadruplexes and genome stability have not been firmly established to date.

Finally, the question arises on whether there is an association between tumors exhibiting genome instability and defective DNA methylation. Genome instability can arise from many types of damage, including to telomeres, centromeres, replication stress and DSBs. A meta-analysis study identified a positive correlation between cancer incidence and DNA methylation at the promoter regions of genes involved in regulating telomere maintenance and regulation [129]. This suggests that cancer cells can alter telomere homeostasis through DNA methylation. Centrosomal defects are observed in breast cancer and result in aneuploidy due to chromosomal instability (CIN) [130]. Since p53 signaling axis is often compromised, p21, its *bonafide* target gene, can no longer inhibit the PCNA-DNMT1 complex from methylating DNA [59]. This results in aberrant DNA methylation, which could contribute to the CIN-phenotype. Thus, these studies highlight increasing evidence connecting defective DNA methylation and genome instability in cancer.

Although much of what we know about DNA methylation and genome stability relies on studies involving DNMT1, it is exciting to consider that this epigenetic mark is reversible. While, this makes DNA methylating inhibitors an attractive target in cancer therapeutics, very little is known about DNA demethylation in the context of the DDR and genome stability. Given that DNA methylation is read by a host of reader proteins (Figure 4), the potential impact of DNA methylation on the binding and function of these proteins in genome integrity pathways is clear but has not yet been studied comprehensively. Our current limited knowledge on DNA methylation and its biological effects on genome maintenance needs to be extended to allow additional forays into therapeutic targeting of these pathways to be made. It is conceivable that DNA methylation inhibitors in combination with other drugs, including those targeting the DDR, or in mutational backgrounds that could provide genetic vulnerabilities to these compounds can offer promising possibilities for cancer treatment (Figure 6). Future studies should focus on understanding canonical and non-canonical roles of DNA methylation in not only gene

expression, but also their roles in maintaining genome integrity. This information has the potential to be leveraged to better identify and treat cancer.

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Abbreviations

5-caC	5-carboxycytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5-mC	5-methylcytosine
AID	activity-induced cytosine deaminase
ALKBH1	alkylated DNA repair protein B homolog
AML	acute myeloid leukemia
AP site	apyrimidinic site
APOBEC	apolipoprotein B mRNA editing complex
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3-related
BER	base excision repair
bZIP	basic leucine-zipper
CGI	CpG island
CPT	camptothecin
DDR	DNA damage response
DNMT	DNA (cytosine-5)-methyltransferase 1
DSB	double-strand break
FDA	Federal Drug Administration
hm^{6}dA	human N^6-hydroxymethyladenine

HR	homologous recombination
IR	ionizing radiation
lncRNA	long non-coding RNA
MBD	methyl-CpG-binding domain
MGMT	O^6-methylguanine-DNA methyltransferase
MLL	mixed lineage leukemia
MMR	mismatch repair
N6AMT1	N^6-adenine-specific DNA methyltransferase 1
NHEJ	non-homologous end joining
PBD	PCNA-binding domain
PCNA	proliferating cell nuclear antigen
PIP	PCNA-interacting protein
R-loop	RNA:DNA hybrid
SAM	S-adenosyl-L-methionine
TDG	thymine DNA glycosylase
TE	transposable element
TET	ten-eleven translocation protein
UHRF1	Ubiquitin-like, containing PHD and RING finger domains 1
UV	ultraviolet

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Summary

- DNA methylation is a reversible epigenetic mark that plays an important role in gene expression and DDRs.
- Exposure to DNA damaging agents can affect DNA methylation patterns, causing mutations like deamination and increased transposon activation. However, it still remains to be determined if loss of DNA methylation patterns impacts the DDR directly, which could result in additional genomic and epigenomic instabilities.
- DNMT1 interacts with the replisome clamp PCNA and its dysregulation results in replication stress and mutations. Testing for roles of DNA methylation directly in replication and repair fidelity is warranted.
- Epigenetic inactivation of tumor suppressor genes due to aberrant methylation contributes toward increased mutations and genome instability.
- DNMT targeting drugs beyond those targeting DNA methylation catalytic activities should be considered.
- Many questions remain about the molecular mechanisms that govern DNA methylation and genome integrity, including the role of DNA demethylases, adenine methylation and readers of methylated DNA in the DDR.

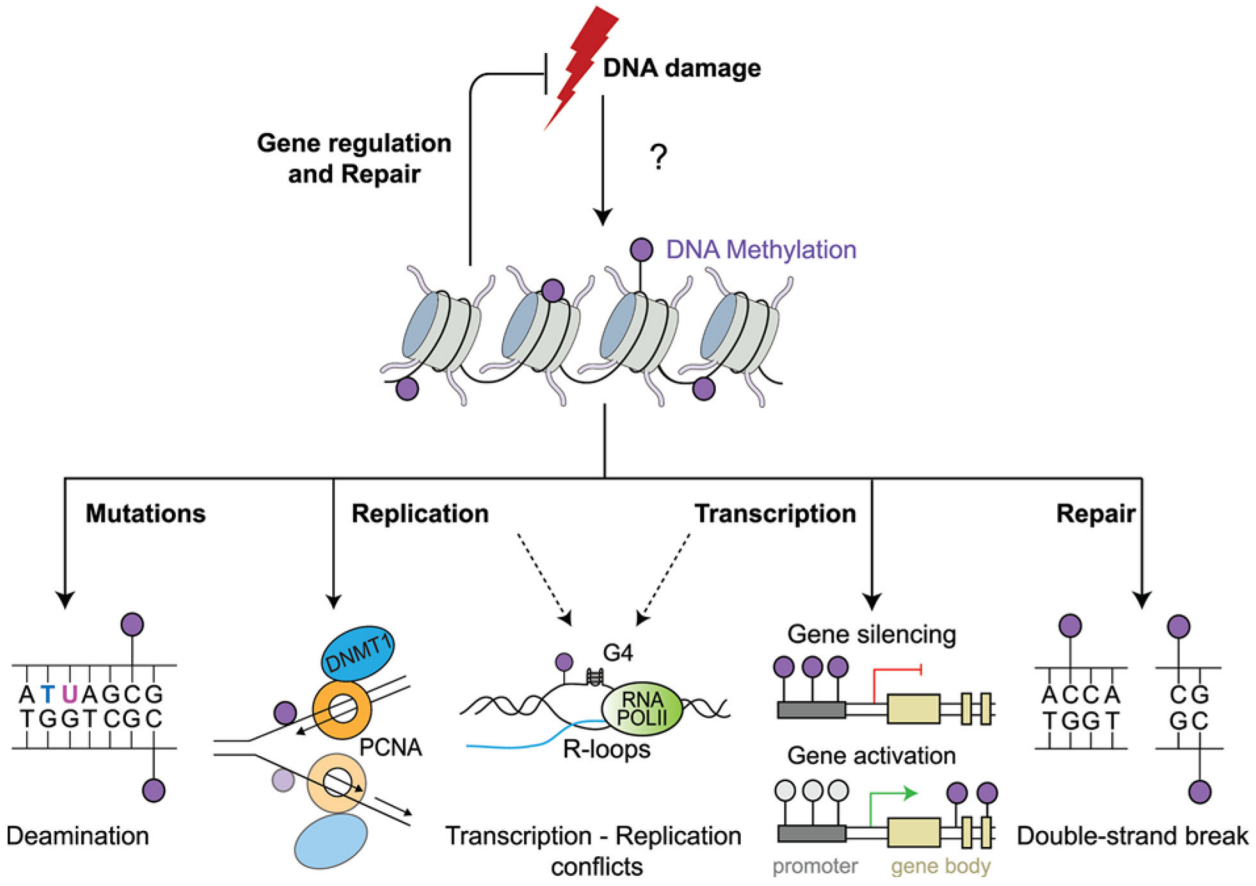

Figure 1. Potential roles of DNA methylation in genome maintenance

A simplified model depicting the putative roles of DNA methylation in the maintenance of genome integrity. DNA is methylated commonly at CpG sites, transposable elements, sites of tissue-specific gene silencing, X-chromosome inactivation and genome imprinting. DNA can undergo spontaneous deamination causing mutations or encounter roadblocks during replication from secondary structures such as R-loops and G-quadruplexes (G4), which may confer aberrant methylation patterns across the genome affecting gene transcription or impacting DNA DSB repair in response to DNA damage. Collectively, DNA methylation has the potential to affect the DDR as illustrated. Likewise,alterations in these pathways could also alter DNA methylation, which warrants consideration.

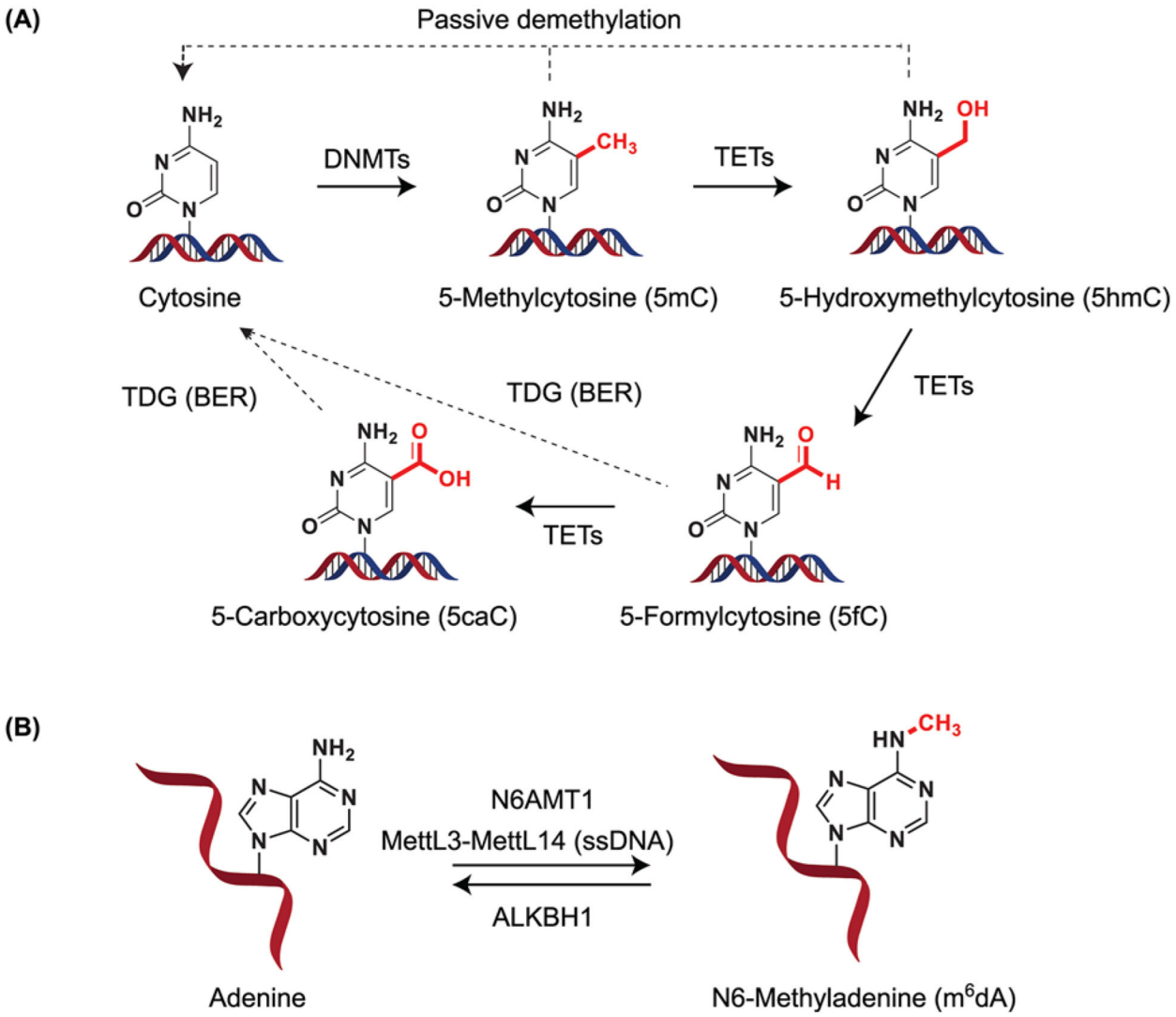

Figure 2. An overview of DNA methylases and demethylases

(**A**) Structural depiction of cytosine methylation to form 5mC. Upon TET activity, 5mC can give rise to 5-hydroxymethylcytosine (5hmC). TETs can iteratively oxidize 5hmC to 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). 5fC and 5caC can be recognized and excised by the action of TDG forming cytosine through BER. (**B**) DNA methylation in adenine. Adenine can be methylated by N6AMT1 in DNA. The m6A RNA methyltransferase MettL3–MettL14 has been suggested to also methylate ssDNA. Demethylation has been suggested to occur via ALKBH1 activity. DNMT - includes DNMT1, DNMT3A, DNMT3B; TETs - TET1, TET2, TET3. Abbreviations: BER, base excision repair; N6AMT1, N^6-adenine-specific DNA methyltransferase 1; TDG, thymine DNA glycosylase.

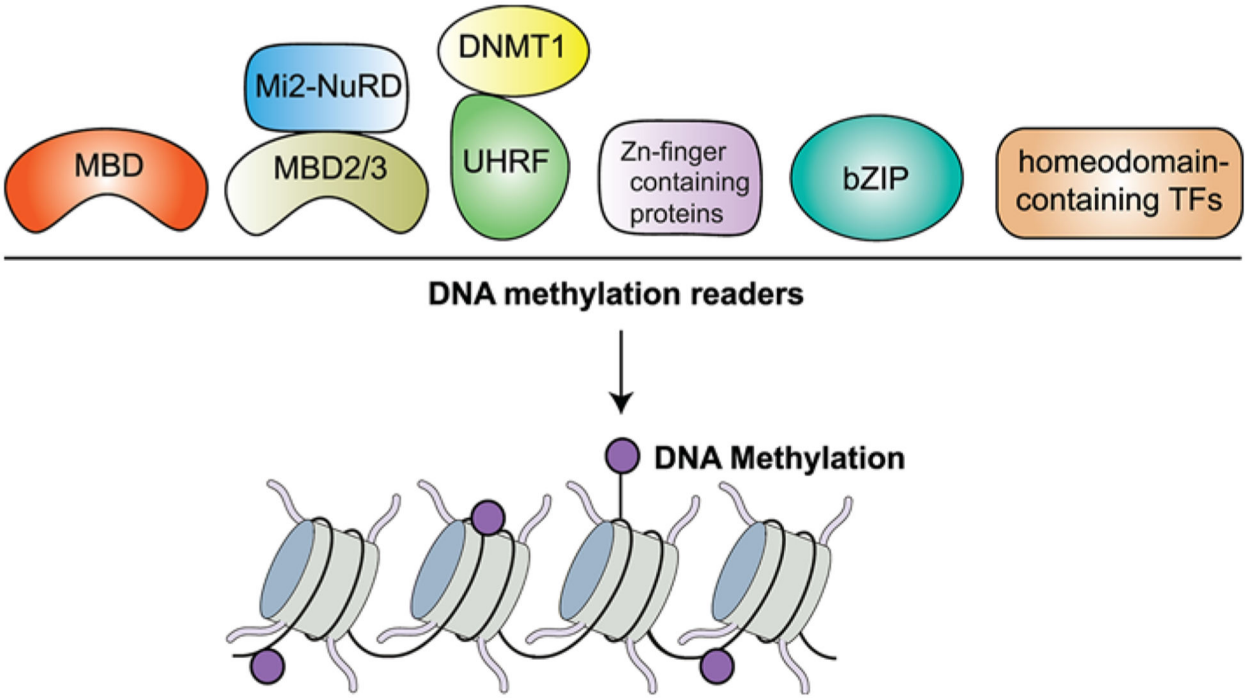

Figure 3. Readers of DNA methylation

DNA methylation is recognized by several proteins which include: MBD, UHRF, Zn finger domain proteins including ZBTB33 (Kaiso), ZBTB4, ZBTB38, CTCF, KLF4, WT1, EGR1, ZFP57, basic leucine zipper-containing TFs (bZIP) and homeodomain-containing TFs.

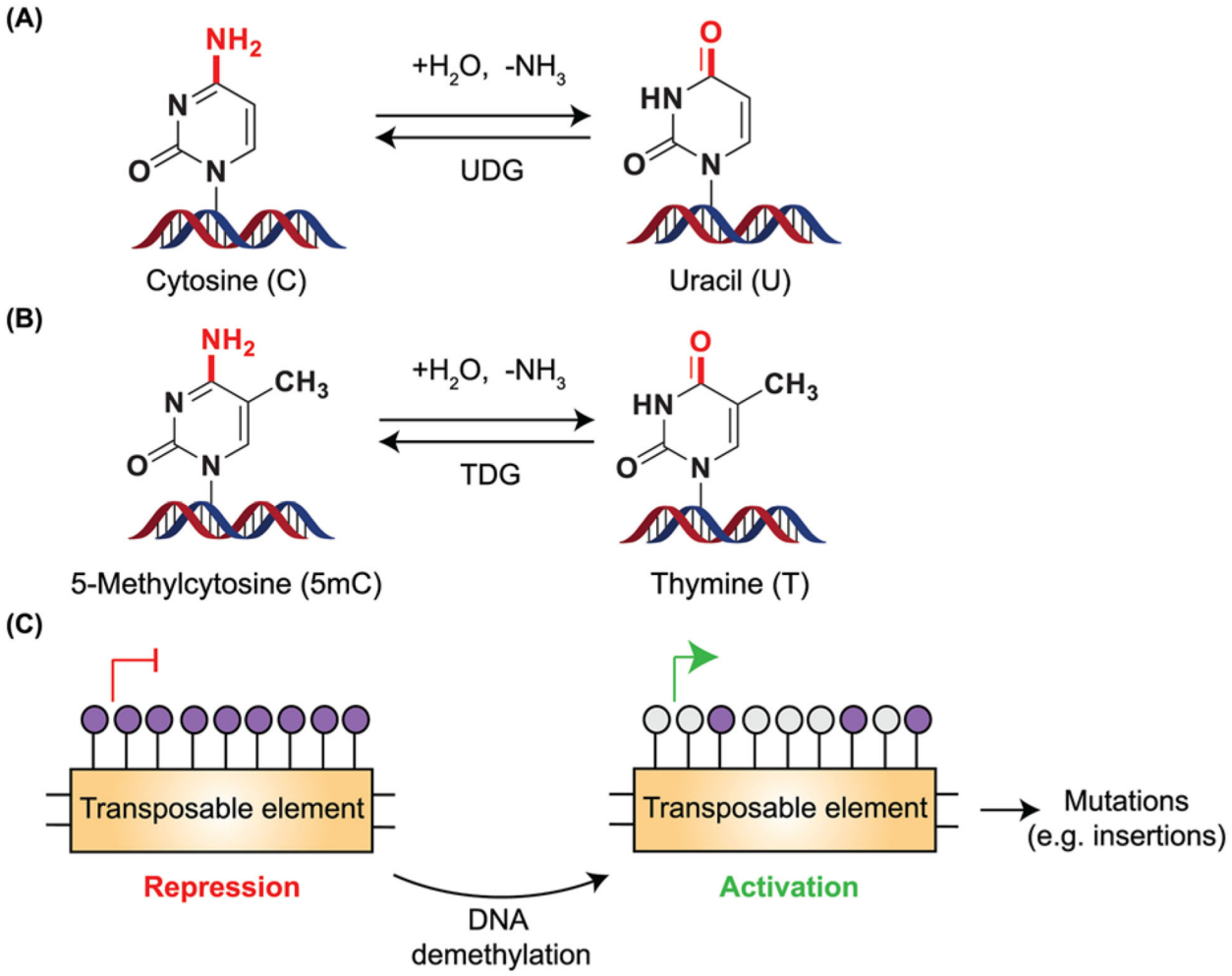

Figure 4. DNA methylation and mutations

(**A**,**B**) Cytosine and 5mC undergo deamination either spontaneously or when exposed to agents that are hydrolytic or alkylating. Hydrolytic deamination (removal of -NH_2 group as ammonia) from cytosine leads to the formation of uracil. Since uracil is not present in DNA, it is readily recognized by UDG, resulting in the substitution of uracil to cytosine. However, when 5mC undergoes deamination, it leads to the formation of thymine, a base normally present within DNA. TDG recognizes and corrects these misincorporated bases but inappropriate repair causes C→T transition mutations within the genome. (**C**) Transposable elements present in the genome are generally silenced by hypermethylation. DNA demethylation leads to the activation of these transposable elements, with the potential to increase mutations including insertions.

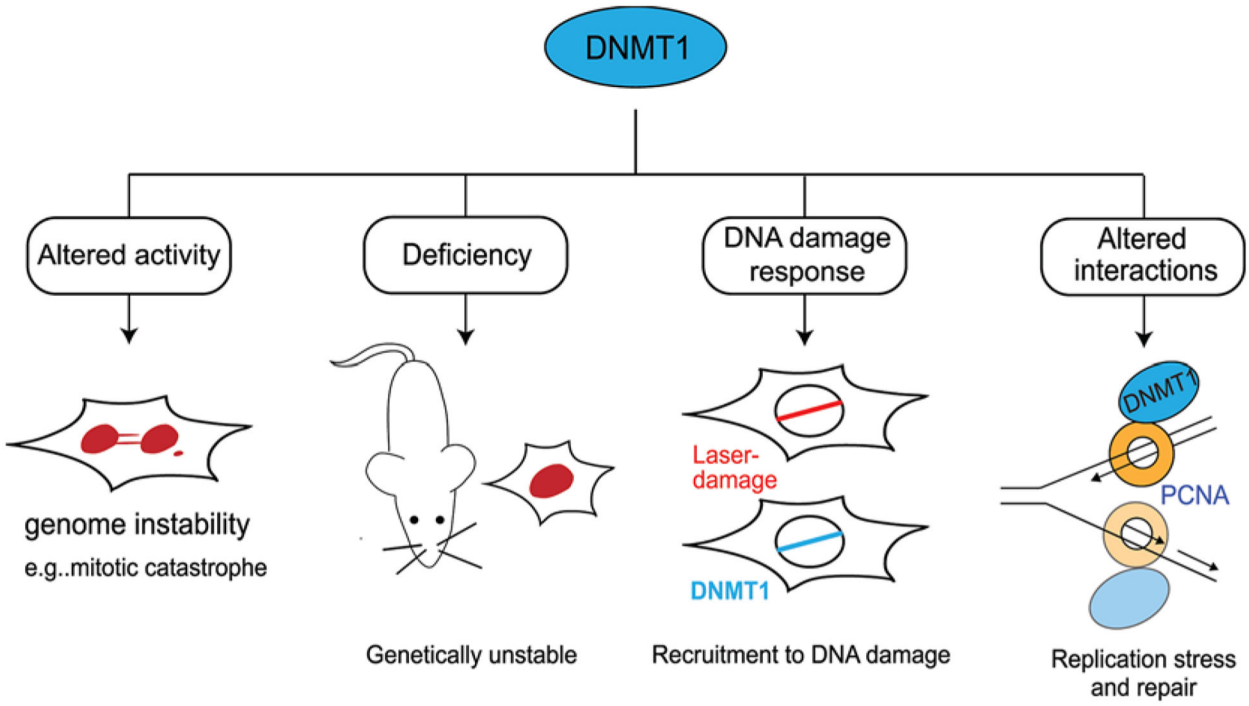

Figure 5. Roles of DNMT1 in genome integrity

DNMT1 plays several critical roles in maintaining genome stability. These include: altered activity of DNA methylation can result in mitotic catastrophe; deficiency of DNMT1 has been found to be genetically unstable; DNMT1 is recruited to sites of laser damage; DNMT1 interacts with the replisome clamp PCNA during DNA replication and repair processes.

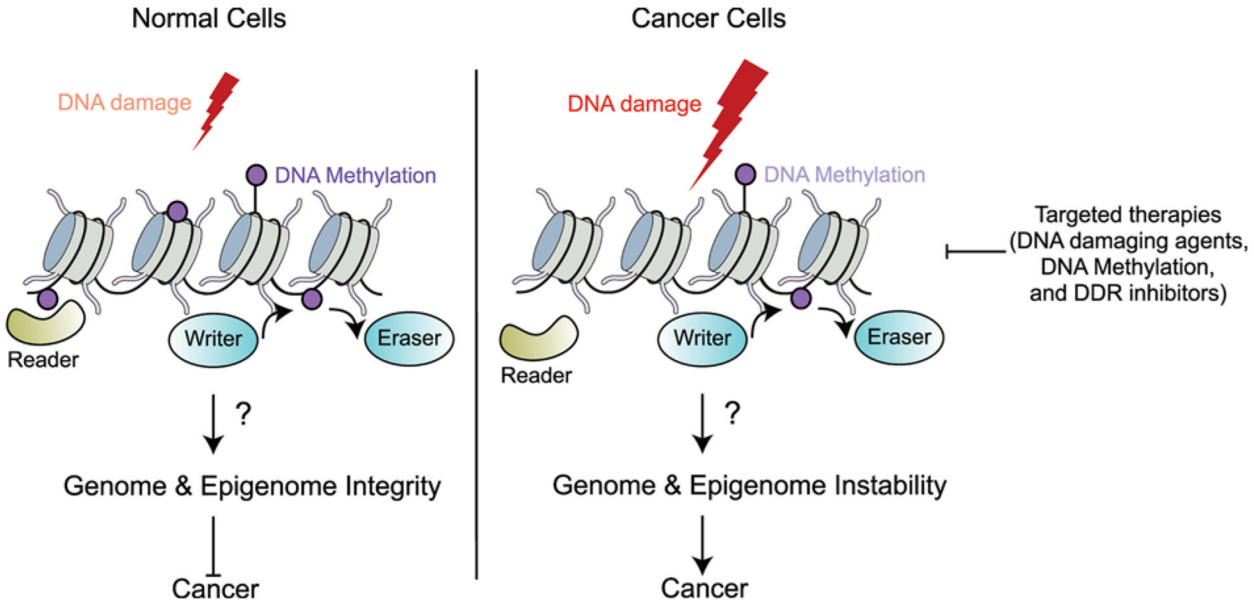

Figure 6. Future perspectives

When normal cells encounter DNA damage, readers, writers and erasers of DNA methylation may contribute to the cellular response to DNA damage via gene regulation, DDRs and repair processes to ensure the maintenance of genome and epigenome integrity. However, in cancer cells, the function of readers, writers and erasers of DNA methylation may be altered. The changes in the methylation landscape could result in genomic and epigenomic instability due to differential gene expression, mutations and endogenous DNA damage, resulting in genome instability, a hallmark of cancer. Increased understanding of the mechanisms surrounding DNA methylation upon DNA damage and maintenance of genome integrity is necessary to extend current therapeutic strategies. Combinatorial treatments of inhibitors of DNA methylation along with DNA damaging agents and drugs targeting the DDR (i.e. PARP inhibitors) could offer promising drug treatment options to target cancer cells with altered DNA methylation patterns.