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Alternative roles for oxidized mCs and TETs

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Abstract

Ten-eleven-translocation (TET) proteins oxidize 5-methylcytosine (5mC) to form stable or transient modifications (oxi-mCs) in the mammalian genome. Genome-wide mapping and protein interaction studies have shown that 5mC and oxi-mCs have unique distribution patterns and alternative roles in gene expression. In addition, oxi-mCs may interact with specific chromatin regulators, transcription factors and DNA repair proteins to maintain genomic integrity or alter DNA replication and transcriptional elongation rates. In this review we will discuss recent advances in our understanding of how TETs and 5hmC exert their epigenetic function as tumor suppressors by playing alternative roles in transcriptional regulation and genomic stability.

Introduction

Cytosine modifications have been implicated in a wide variety of biological processes such as maintenance of pluripotency, development and differentiation of mammalian cells [1]. 5-methylcytosine (5mC) is the most abundant CpG modification in the mammalian genome and is typically associated with gene silencing [2]. The discovery of the Ten-Eleven-Translocation (TET) family of enzymes and their ability to generate oxidative products of 5mC (oxi-mCs) have led to intense investigation into the role these modifications play in reversing DNA methylation signatures and the regulation of transcriptional activity. Recent evidence points towards additional roles for TETs and oxi-mCs in protecting the genome from the accumulation of mutations and chromosomal lesions that may predispose cells to malignancy.

TET-mediated DNA oxidation

The TET proteins (TET1-3) are a family a α-ketoglutarate (α-KG) and Fe^{2+}-dependent dioxygenases that catalyze the hydroxylation of 5-methylcytosine (5mC) in the mammalian genome to generate 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-

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carboxylcytosine (5CaC) through iterative oxidation reactions [3–6] (Figure 1). The oxidative products of 5mC (oxi-mCs) can exist as stable modifications in the genome or as transient modifications that provide a trigger for DNA demethylation [3,5,7,8]. Passive dilution of 5mC can occur during DNA replication if DNA methyltransferase 1 (DNMT1), which normally targets hemimethylated DNA, is unable to recognize 5hmC [9]. The TET proteins are also able to promote DNA demethylation by triggering base excision repair (BER) of oxi-mCs (5fC or 5caC) [10] or 5hmU generated by deamination of 5hmC [11]. Research into how these modifications shape the epigenetic landscape has been fuelled by the discovery that their dysregulated abundance or loss of normal patterning is a hallmark of cancer.

5hmC and TETs are tumor suppressors

Decreased expression of TET proteins and loss of 5hmC has been documented in multiple tumor tissues [12–14] and mutations or deletions in *TET* genes are prevalent in human cancer genomes. Of the three family members, *TET1* and *TET3* are most frequently mutated in solid tumors such as cutaneous squamous cell carcinoma, melanoma and colorectal cancer [15–17]. *TET2* is primarily mutated in hematopoietic malignancies and is one of the most frequently mutated genes in patients with myelodysplastic syndrome and acute myeloid leukemia (AML) [18–22].

Functional and structural studies using TET2 mutant proteins such as those found in AML patients have revealed that truncation or mutation of the catalytic domain affects the ability of the enzyme to bind Fe^{2+} or α-KG, leading to impaired oxidation of 5mC and DNA hypermethylation [10,23–25]. Isocitrate dehydrogenase (*IDH1/2*) mutations are also widespread in patients with AML [26] or glioma [27] and generate 2-hydroxyglutarate (2-HG) instead of α-KG that inhibits TET function [18,26,28]. In addition, mutation in the Wilms' tumor 1 gene (*WT1*), a DNA-binding partner of TET2, also blocks 5hmC generation due to impaired DNA recruitment [29,30]. Mutations or deletions in *IDH1/2, WT1* and *TET1-3* tend to be mutually exclusive in the majority of tumors [15–17,26] and in AML have been shown to drive overlapping aberrant DNA hypermethylation signatures [18,29–31]. Given that decreased expression or mutation of *TET* genes confers a poor prognosis in multiple cancers [22,32,33], understanding how loss of 5hmC and aberrant DNA methylation in the genome affects tumor biology is an important research objective.

TETs and 5hmC in the regulation of gene expression

A great deal of focus has been placed on studying the role of 5hmC and TETs in transcriptional regulation given the historical association of DNA methylation with gene silencing [2]. All TET proteins bind preferentially at transcriptional start sites (TSSs) and promoters with affinity that positively correlates with CpG density [34–39]. TET1 plays a dual role, activating or repressing its direct target genes [34–36] whereas TET2 binding at promoters positively correlates with gene expression [38]. However, recent studies have shown that TET2 can also act as a negative regulator of lineage specific genes [40]. These findings are consistent with genome-wide mapping studies that show 5hmC localization within gene bodies and promoter regions of both active and repressed genes [35,36,41,42].

5hmC enrichment has also been observed at active enhancers and is depleted upon loss of TET function [43,44]. 5fC and 5caC are even more abundant than 5hmC at poised and active enhancers and promoter TSSs compared to gene bodies [45–47]. Overall these studies would suggest that TETs, by modulating the balance between inter-genic and intra-genic oxi-mCs, play an important role in dictating the transcriptional outcome of bivalent genes.

Although not experimentally proven, TET proteins may also regulate gene expression by altering chromosomal architecture through its ability to modulate 5mC. The insulator protein, CCCTC-binding factor (CTCF) is inhibited by 5mC from binding at gene bodies [48] and has been shown to require TET-mediated oxidation of 5mC to regulate alternative splicing [49]. TETs could also regulate chromosomal architecture by protecting large undermethylated genomic regions known as DNA methylation “canyons” from hypermethylation [50]. 5hmC enrichment at canyon edges and the aberrant expression of canyon-associated genes in cancer implicates an important role for TETs and 5hmC in their maintenance [51].

TETs and oxidized mC in genomic integrity

The most common single nucleotide polymorphisms (SNPs) and somatic mutations found in cancer genomes is the transition of C to T in the context of CpG dinucleotides [52,53]. This is attributed to an increased tendency for spontaneous deamination of 5mC compared to C [54,55] and the differential recruitment of error-prone repair enzymes. Deamination of an unmethylated C or 5hmC generates U:G or 5hmU:G mismatches that are efficiently repaired by thymine and uracil DNA glyosylases, TDG and SMUG1 [11,56]. 5mC deamination generates T:G mismatches that can recruit TDG but also the error-prone mismatch repair (MMR) complex, MutSα [57,58]. Cytosine deaminases (AID and APOBEC1-3) also have increased activity on 5mC than 5hmC [59,60]. Consequently, the less mutagenic variant of cytosine, 5hmC, may provide protection from deamination-mediated C-T transitions in the mammalian genome. Comparing base-resolution maps of 5mC and 5hmC in normal human brain, kidney and myeloid cells with cytosine mutations in tumors from these tissues, it was indeed shown that 5hmC sites exhibit a 50% decrease in C-T mutation frequency than 5mC and a mutation load similar to that of unmodified cytosines [61]. Another study also reported a minor reduction in C-T transition SNP rates at 5hmC compared to 5mC in mammalian genomes [54].

Interestingly, a higher rate of C-G transversion at 5hmC sites has also been reported in human and mouse ESCs [54]. These transversions may be a consequence of breakdown in repair of the less stable 5fC or 5caC modifications. Unlike 5hmC:G base pairs, TDG recognizes and excises 5fC/5caC:G mismatches [6,62,63] and 5caC:G pairs are bound as strongly as T:G mismatches by the MMR complex [62]. In addition, these oxi-mCs could be recognized by different error-prone DNA polymerases, recruit unique DNA damage response protein proteins or depending on the nucleotide context may differ in their efficiency of repair. Ultimately, in cancer genomes, C-G transversions are orders of magnitude less frequent than C-T transitions [53,55] and 5hmC is vastly more abundant than 5fC and 5caC [4,6,63]. In protecting the genome from DNA hypermethylation, TET proteins

and 5hmC may play a dual role in maintaining genomic integrity by limiting the rate of cytosine loss through deamination of 5mC.

DNA repair and damage sensing by oxi-mCs

The placement of 5hmC at the precipice between a stable epigenetic modification and the formation of 5fC and 5caC makes it an ideal intermediate for the recruitment of DNA damage sensing and repair enzymes. In HeLa cells, 5hmC but not 5mC was recently found to co-localize by immunostaining with γH2AX, 53BP1 and RAD51 foci in what appeared to be G1-specific 53BP1 nuclear bodies [64] (Figure 2). In this study, the co-localization at sites of DNA damage was dependent on TET2 and exacerbated by genototoxic insults such as aphidicolin treatment to induce double strand breaks (DSBs). 5hmC foci were never seen without 53BP1 staining, suggesting that damage-induced 5hmC is removed along with 53BP1 during S phase [64,65]. Interestingly, 5mC, 5fC or 5caC staining was absent at DNA damage foci providing further evidence that 5hmC at sites of DNA damage is a stable epigenetic mark [64]. TET proteins and 5hmC are however not essential for the recruitment of DNA repair proteins given studies performed in *Tet*-triple knockout (*Tet*-TKO) mESCs, showed that γH2AX foci still formed and could be resolved in response to aphidicolin treatment [64]. Instead, at low dose aphidicolin treatment, which mimics replication stress [65], a greater frequency of chromosome segregation defects were found to occur in *Tet*-TKO ESCs [64]. *Tet*-TKO mESCs have also been reported to exhibit increased telomere–sister chromatid exchange [43], a mechanism of homologous recombination (HR) and telomere elongation independent of telomerase [66]. A failure to adequately repair DNA damage sustained during S phase can lead to under-replicated regions that interfere with the separation of chromosomes in mitosis [67]. Accumulation of γH2AX also occurs in the absence of DSBs to regulate cell cycle progression by inhibiting DNA replication [68,69]. In this context the overexpression of TET proteins in NIH-3T3 cells has been shown to induce 5hmC, accumulate γH2AX and delay DNA replication [70]. Given that genomic stability relies on DNA replication fidelity during S phase and correct segregation of sister chromatids during mitosis, the role of oxi-mCs in these highly coordinated cellular processes should be investigated further.

Loss of TET function and genomic instability in cancer

Reducing 5hmC levels below a critical threshold may lead to overt genomic instability in adult cells. Recent studies in mouse models have shown that TET-deficiency plays a causal role in genomic instability and disease progression. Our laboratory discovered that *Tet1* deficiency causes decreased expression of DNA repair genes and a spontaneous increase in γ-H2AX foci in developing B cells [71]. B cell progenitors also exhibit aberrant self-renewal and mice spontaneously develop B cell lymphoma upon advanced age [71].

Combined *Tet2/Tet3* loss in mouse hematopoietic cells drives an aggressive leukemia that correlates with the spontaneous accumulation of γH2AX and impaired DNA damage repair (DDR) in myeloid progenitors [72]. Gene expression differences in both HR and non-homologous end-joining (NHEJ) repair pathways were also observed in *Tet2/3* DKO immature myeloid cells [72]. It is not clear however whether these expression changes are

the cause or consequence of defects in DNA damage responses. These studies suggest that Tet2 and Tet3 functionally overlap to maintain a threshold of 5hmC that protects developing myeloid cells from genomic instability as neither KO alone causes spontaneous γH2AX accumulation [72]. Interestingly, TET-deficient hematopoietic stem cells (HSCs) do not show defects in DDR [72,73]. This may be because loss of TET function and the subsequent dilution of 5hmC in the genome require several rounds of DNA replication and the replicative history of HSCs is much younger than progenitors. If this is the case, long-lived HSCs with increased rounds of replication may exhibit greater accumulation of DNA damage in TET-deficient mice.

Readers of oxi-mCs

5hmC could serve as a stable intermediate not only for DNA demethylation but also for the recruitment of chromatin or transcriptional regulators and repair proteins in times of DNA damage or replication stress. 5hmC and 5mC are indistinguishable to DNA polymerases [74] however in a screen to find interacting proteins, these marks exhibit unique reader profiles, and overlap in their recruitment of only a small number of transcriptional regulators, such as MeCP2 and Uhrf1 [75]. DNA glycosylases (Mpg and Neil3) and helicases (Recq1) are uniquely recruited to 5hmC and not 5mC in mESCs whereas 5fC and 5caC recruit a large number of DNA repair proteins, including BER (Neil1, Neil3, and Mpg) and MMR (Msh3 and Exo1) factors [75]. Of all the oxi-mCs, 5fC is the only modification that alters the structure of the DNA double-helix, characterized by helical unwinding, suggesting that recognition of the altered DNA conformation rather than the modified base itself might trigger biological events [76]. This finding could explain why the enriched binding of DNA repair-associated proteins is most pronounced for 5fC [75].

Oxidation of 5hmC may also control the rate of DNA replication and RNA transcription (Figure 2). 5caC is recognized by the proofreading DNA polymerase δ causing a brief stalling at this modified base that may delay DNA synthesis [62]. 5fC and 5caC have also been reported to induce a transient pause in the RNA polymerase II elongation complex that may regulate the rate of transcription as well as promote alternative exon usage [77,78]. TET enzymes also target thymine for oxidation, generating 5hmU, which when paired with adenine is selectively repaired by SMUG1 [79] and attracts its own complement of specific protein readers [75,80]. Given the large number of proteins that recognize oxi-mCs, a combination of diverse biological processes have the potential to regulate gene expression and DNA fidelity by these modifications.

5hmC and asymmetric epigenetic inheritance

5mC sites are highly symmetric, where 99% of methylated CpGs are found to be methylated on both strands [81]. 5mC is copied onto newly synthesized DNA by DNMT1 however 5hmC maintenance, through the action of TET enzymes on newly synthesized DNA strands, has not been reported. Recent sequencing efforts have discovered that 5hmC is distributed asymmetrically in the genome.

Initial studies of whole-genome 5hmC mapping in bulk cell populations led to conflicting reports on the existence of 5hmC strand bias in mouse ESCs [82,83]. A newly described method for genome-wide, strand-specific, single-cell 5hmC sequencing has now confirmed that 5hmC varies in abundance on the two DNA strands of a given chromosome up to 10-fold [84]. Based on the observation that 5hmC marks are passively lost during replication [73] Mooijman et al (ref [84]) hypothesized that differences in strand age between the plus and minus strands of a chromosome could be a potential mechanism for generating the observed 5hmC strand bias [84]. Assuming that cells divide symmetrically, with random chromosome segregation, each of the two daughter cells would inherit one strand of an original mother chromosome. Comparing the distribution of the strand bias between oocytes and single cells from haploid two-cell embryos, the oocytes were shown to have no significant strand bias, whereas single cells from two-cell embryos displayed strong strand bias, with each sister cell receiving an old strand. This heritable 5hmC bias allowed the authors to develop a model to infer sister-cell relationships and cell lineage reconstruction in sets of 4-cell embryos [84]. Asymmetric inheritance of 5hmC has also been observed by immunofluorescence in an adult stem cell model of non-random chromosome segregation [85]. However, the observations from single-cell sequencing of 5hmC in embryos suggests that asymmetric 5hmC inheritance is a general phenomenon and not exclusive to asymmetric cell divisions. The asymmetric inheritance of 5hmC upon DNA replication may serve as a source of chromosome-wide epigenetic memory and has also been observed with histones [86]. Strand-bias of 5hmC may dictate asymmetric distribution of gene regulation or DNA fidelity of template strands upon cell division. Whether this is mediated via the strand-specific recruitment of oxi-mC readers or DNA repair machinery is not yet known.

Conclusions and Future Directions

The tumor suppressive role of TETs and oxi-mCs and their association with the prevention of aberrant DNA hypermethylation extends beyond our conventional view of gene regulation. Unmodified and modified cytosines exhibit different rates of mutation and elicit unique DNA repair responses. The absence of TETs or 5hmC may limit the recruitment of many of the readers of oxi-mCs that are intimately involved in DNA damage repair independently of their role in transcriptional regulation. Steric effects of oxi-mCs in genomic topology could also influence the rate of DNA replication or gene transcription. 5hmC strand bias may be another mechanism by which TETs regulate asymmetric gene expression or DNA repair to instruct cell fate, drive differentiation and protect cells from replication induced DNA damage. DNA hypermethylation and deficient DNA repair may therefore predispose cells to the acquisition of oncogenic mutations independently of the transcriptional role for TETs and 5hmC. Future studies should continue to explore, at single-base resolution, the correlation between mutation frequency, oxi-mC and asymmetric distribution of these modifications in genome-wide sequencing studies to shed light on how TETs and oxi-mCs protect cells from malignant transformation.

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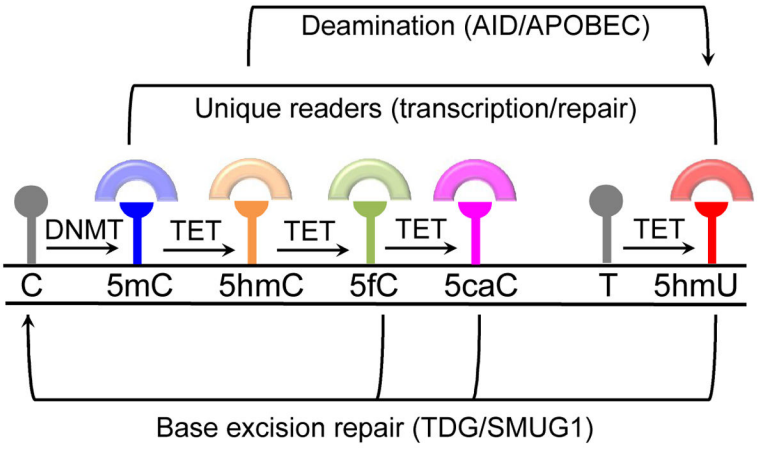

Figure 1. TET-mediated DNA oxidation products

DNA methyltransferases (DNMT) and a methyl group to cytosine (C) forming 5-methylcytosine (5mC) that can be converted by TET proteins to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) through iterative oxidation reactions. The oxidative products of 5mC (oxi-mCs) can exist as stable modifications in the genome, or as transient modifications that provide a trigger for DNA demethylation. The TET proteins are able to promote DNA demethylation by triggering base excision repair (BER) via DNA glycosylases (TDG/SMUG1) that target oxi-mCs (5fC or 5caC). 5hmU generated by deamination of 5hmC (via AID/APOBEC deaminases) or by TET-mediated oxidation of thymidine (T) can also trigger BER. Each oxidation product of TET can recruit unique readers (colored semi-circles) that may exert different biological functions in response to the presence of the DNA modification in the genome.

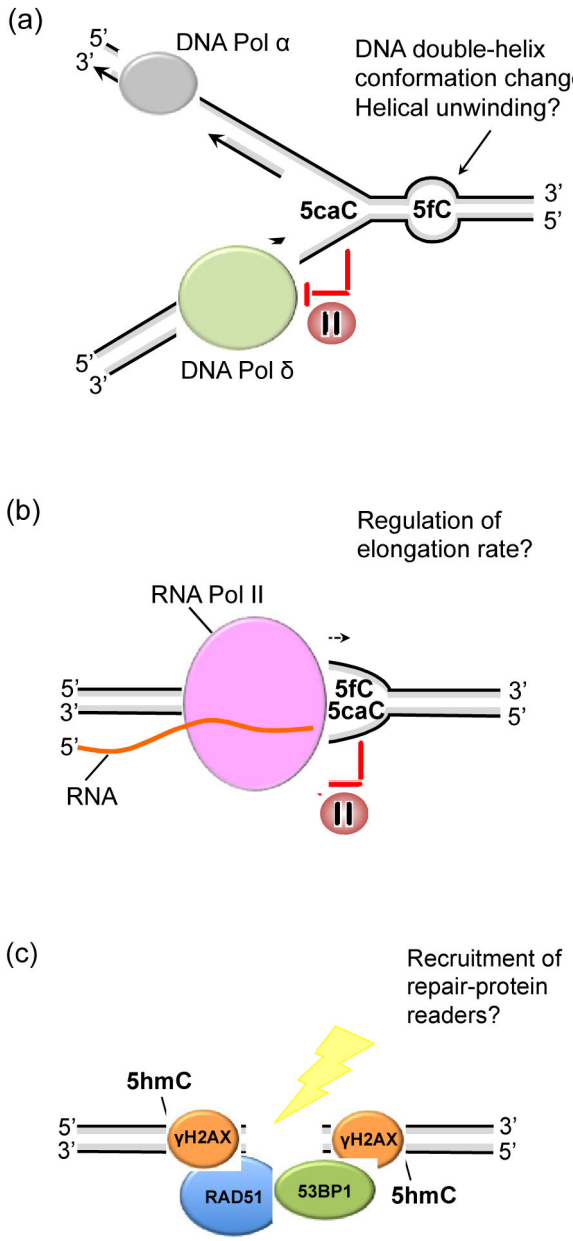

Figure 2. Alternative roles for oxi-mCs in replication, transcription and repair

A) 5caC is recognized by the proofreading DNA polymerase δ causing a brief stalling at this modified base that may delay DNA synthesis. 5fC alters the structure of the DNA double-helix, characterized by helical unwinding, suggesting that recognition of the altered DNA conformation rather than the modified base itself might trigger biological events. **B)** 5fC and 5caC have been reported to induce a transient pause in the RNA polymerase II elongation complex that may regulate the rate of transcription as well as promote alternative splicing. **C)** 5hmC co-localizes with γH2AX, 53BP1 and RAD51 at sites of DNA damage and double strand breaks.